Influence of IFN-β on the life span and apoptosis of tumor infiltrating neutrophil granulocytes

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von **Lisa Christin Andzinski**

aus Wolfenbüttel

1. Referent: **Prof. Dr. Dirk Heinz**

2. Referent: **Prof. Dr. Michael Steinert**

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Für meine Liebsten

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1. Abstract

Efficient immunosurveillance is a major factor in protecting the individual against cancer. Components of the innate immune system like neutrophilic granulocytes and type I IFNs were demonstrated to significantly influence this complex process by altering tumor angiogenesis and growth. Consistently, many neutrophil activities seem to be controlled by endogenous IFN-β. Nevertheless, little is known about its detailed impact on life span and polarization of such cells. Moreover, whether and how type I IFN is induced in the tumor context and from which cellular sources it is derived is not clear. These issues were addressed in the present study.

Using different transplantable tumors in an IFN-β reporter mouse model, it could be demonstrated for the first time that IFN-β is induced in solid tumors. This induction is mediated in a STING-IRF3/5 dependent manner and tumor infiltrating myeloid dendritic cell were identified as a main cellular source of this cytokine.

Furthermore, the present results provide evidence that endogenous IFN-β is regulating apoptosis, maturation and turnover of pro-angiogenic tumor infiltrating neutrophils by influencing both, the extrinsic as well as the intrinsic apoptosis pathways. Accordingly, the life span of tumor associated neutrophils (TANs) was remarkably prolonged in tumor bearing *Ifnb1*$^{-/-}$ mice, compared to wild type (WT) controls. Lower expression of Fas, reactive oxygen species, active caspase 3 and 9 as well as a change in expression ratio of pro- and anti-apoptotic members of the Bcl-2 family were observed under such conditions. In addition, the major apoptosome constituent Apaf-1 was downregulated. In line with inhibition of apoptosis and increased neutrophil longevity in the absence of endogenous IFN-β, a strong enhancement of G-CSF expression and PI3 kinase phosphorylation was detected.

During the present study substantial evidence was accumulated supporting the role of endogenous IFN-β as factor responsible for the induction of an N1 anti-tumor neutrophil polarization. Hence, a significant down regulation of N1 associated neutrophil features such as ICAM1 expression, TNF-α production and tumor killing capacity were observed in *Ifnb1*$^{-/-}$ mice, compared to WT controls. Of note, exogenous IFN-β therapy of tumor bearing

WT animals significantly influenced neutrophil polarization at the primary tumor site and in the pre metastatic lung, inducing a N1 anti-tumor neutrophil phenotype.

Taken together, the present work provides important insight into the molecular mechanisms underlying type I IFN-mediated cancer immunosurveillance via the polarization of tumor associated neutrophilic granulocytes.

2. Introduction

Despite substantial research effort, cancer is still the second most common cause of death in the industrialized world with rising incidence. Therefore, understanding the molecular mechanisms of this disease is of utmost importance for the development of new therapies. Especially in the light of several recent findings, the innate immunosurveillance against cancer needs to be carefully investigated.

2.1. The type I IFN system

Type I interferons (IFNs) were first described more than 50 years ago as factors responsible for the phenomenon of viral interference (Isaacs and Lindenmann, 1987). This monomeric family of cytokines includes at least 12 subtypes of IFN-α, IFN-β, IFNε, IFN-κ and IFN-ω (Pestka et al., 2004) in mice and humans. All these type I IFNs signal via a common cell surface receptor IFNAR that is virtually present on every cell type (Pestka et al., 2004). Therefore, they are involved in the regulation of numerous biological processes central to innate and adaptive immunity.

2.1.1. Induction of type I IFNs

The expression of type I IFNs, such as IFN-β, is rapidly induced after recognition of different pathogen associated molecular patterns (PAMPS) or danger associated molecular patterns (DAMPs) by host pathogen recognition receptors (PRRs) including the Toll-like receptors (TLRs), RIG-like receptors (RLRs) or cytosolic DNA receptors (CDRs) (Keating et al., 2011). Besides viral and bacterial RNA or DNA, viral glycoproteins or bacterial cell surface compounds like lipopolysaccharide (LPS), also host DNA is capable to induce significant type I IFN responses (Fuertes et al., 2013). The signaling pathways that lead to IFN induction differ, depending on the stimulus and the responding cell type. Nevertheless, they involve common signaling molecules such as IFN regulatory factors (IRFs) or nuclear-factor-κβ, that bind to the *IFNA* as well as the *IFNB* promoters to initiate gene expression as depicted in figure 2.1. (González-Navajas et al., 2012).

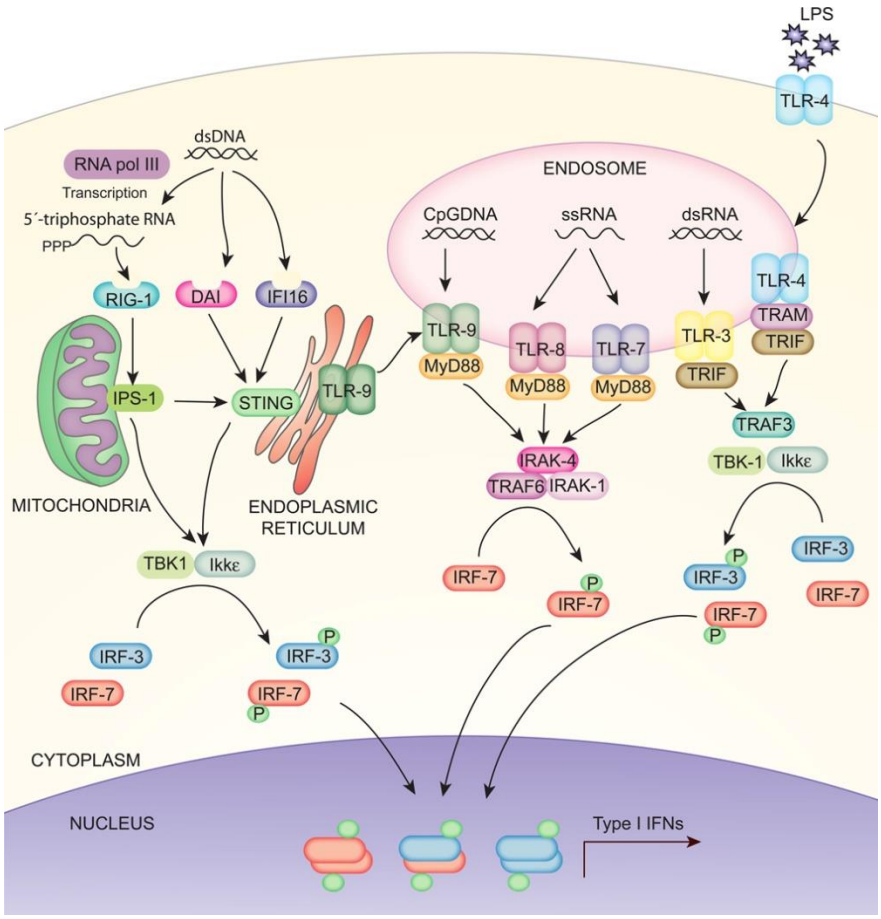

Figure 2.1. Major pathways of IFN induction. CDRs, RLRs and TLRs are the main PRRs that induce type I IFN responses. They signal via STING, IPS-1/MAVS, TRIF or MyD88, the kinase TBK1 and IRFs and in the end lead to the expression of type I IFN genes upon the recognition of potentially dangerous substances like dsDNA, CpGDNA, ss/ds RNA or LPS. (Fuertes et al., 2013)

TLRs are the key sensors for microbial invasion in mammals and activate innate defense mechanisms crucial for host survival (Medzhitov, 2001). In mice and humans six TLRs are known to induce type I IFNs (Noppert et al., 2007). TLR2, TLR3, TLR4, TLR7, TLR8 and TLR9 sense various bacterial and viral products and signal via TIR-domain-containing adaptor protein inducing IFN (TRIF) or myeloid differentiation-associated primary-response protein 88 (MyD88) to induce a type I IFN response (Monroe et al., 2010; Dietrich et al., 2010).

However, mice can also produce type I IFNs in response to RNA and DNA ligands independently of TLR signaling. These TLR-independent pathways include PRRs like retinoic-acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and DNA-dependent activator of IRFs (DAI) (González-Navajas et al., 2012). RLRs like

RIG-I, on the one hand, signal via the adaptor protein MAVS (mitochondrial antiviral signaling protein, Cardif, IPS-1) and CDRs like DAI activate the endoplasmic reticulum-resident protein STING (stimulator of IFN genes). Recently cGAS has been described as additional sensor for intracellular DNA. Upon binding of DNA this enzyme generates a second messenger (cyclic GMP-AMP) which also activates STING (Cai et al., 2014). Both adaptor proteins are known to activate the TANK-binding kinase 1 (TBK1) which subsequently phosphorylates IRF3 and IRF7 (Keating et al., 2011). Upon activation, IRF3 forms homodimers or heterodimers with IRF7, translocates to the nucleus and activates the transcription of type I IFN genes. As a rule, IFN-β is produced first initiating a cascade of IFN-α via autocrine and paracrine loops (Lienenklaus et al., 2008). Here, constitutively expressed IRF-3 is primarily involved in the induction of IFN-β in the early phase of the response, whereas IRF-7, the expression of which is induced by IFN-β, functions in the later phase to induce IFN-αs as well as additional IFN-β (Taniguchi and Takaoka, 2002). Only plasmacytoid dendritic cells (pDCs) remain an exception and can produce large amounts of IFN-α without prior presence of IFN-β (Barchet et al., 2002). In addition to IRF-3 and 7, other IRFs are involved in type I IFN induction and regulation, e.g. IRF-5 is crucial in MAVS-dependent IFN activation in myeloid dendritic cells (mDCs) (Lazear et al., 2013). Interestingly, the activation of IRF5 seems to be very cell type specific and recently multiple IRF5 splice variants with differential cellular localization and regulation have been identified, further adding to the complexity of type I IFN induction and signaling (Mancl et al., 2005). Besides IRF3, IRF5 and IRF7 that positively regulate type I IFN induction also negative regulators are known, namely, IRF2, IRF4 and IRF 8 (Noppert et al., 2007; Pelka and Latz, 2013).

2.1.2. Signaling pathways of type I IFNs

All type I IFNs signal via a common IFN-α/β receptor (IFNAR), that is ubiquitously expressed on most cells in mice and men. It consists of two different subunits, IFNAR1 and IFNAR2 (Honda and Taniguchi, 2006), and is constitutively associated with Janus kinase 1 (JAK1) and non-receptor tyrosin kinase 2 (TYK2) (Platanias, 2005a). IFNAR engagement results in the activation of these kinases that subsequently phosphorylate and activate different signal transducer and activator of transcription (STAT) family members. In the vast

majority of cells this includes STAT1, STAT2, STAT3 and STAT5. STAT4 and STAT6 however, can likewise be activated by type I IFN signaling in lymphocytes (Gonzalez-Navajas et al., 2012).

Upon activation, STAT1 and STAT2 recruit IRF9 and form a complex called IFN-stimulated gene factor 3 (ISGF3). This complex migrates to the nucleus and induces the expression of IFN-stimulated genes (ISGs) via binding to IFN-stimulated response elements (ISREs) in the promoter regions of the different ISGs (Platanias, 2005b). Besides this classical pathway, STAT1 or STAT3 homodimers can be formed and bind to IFN-γ-activated site (GAS) enhancer elements or STAT3-binding elements (SBEs) to promote ISG expression. In addition to STAT1, 2 and 3, other STAT family members were reported to mediate type I IFN signaling in a cell type specific manner. In general, the variance in STAT expression and its dependence on cell type, activation and differentiation state play a major role in the regulation of the diverse outcomes of IFNAR signaling (Hervas-Stubbs et al., 2011).

Moreover, there is evidence for STAT-independent type I IFN signaling pathways, transduced via mitogen activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), NF-κB, Ras-related protein 1 (Rap 1) or Insulin receptor substrate 1 and 2 (IRS 1/2). An overview of these various IFNAR signaling pathways is depicted in figure 2.2. Interestingly, MAPK, PI3K and NF-κB pathways involve immunoreceptor tyrosine-based activation motives (ITAMs) used by immunoreceptors such as the T cell or B cell receptor and some FC receptors. This indicates a cross-talk among IFNAR and multiple receptors in immune cells (Hervas-Stubbs et al., 2011).

Finally, several negative regulators of IFNAR signaling help to fine tune the whole system, including suppressors of cytokine signaling (SOCS) 1 and 3 (Song and Shuai, 1998), the protease UBP43 (Ritchie et al., 2004) or different members of the PIAS (protein inhibitor of activated STAT) family (Liu et al., 2004).

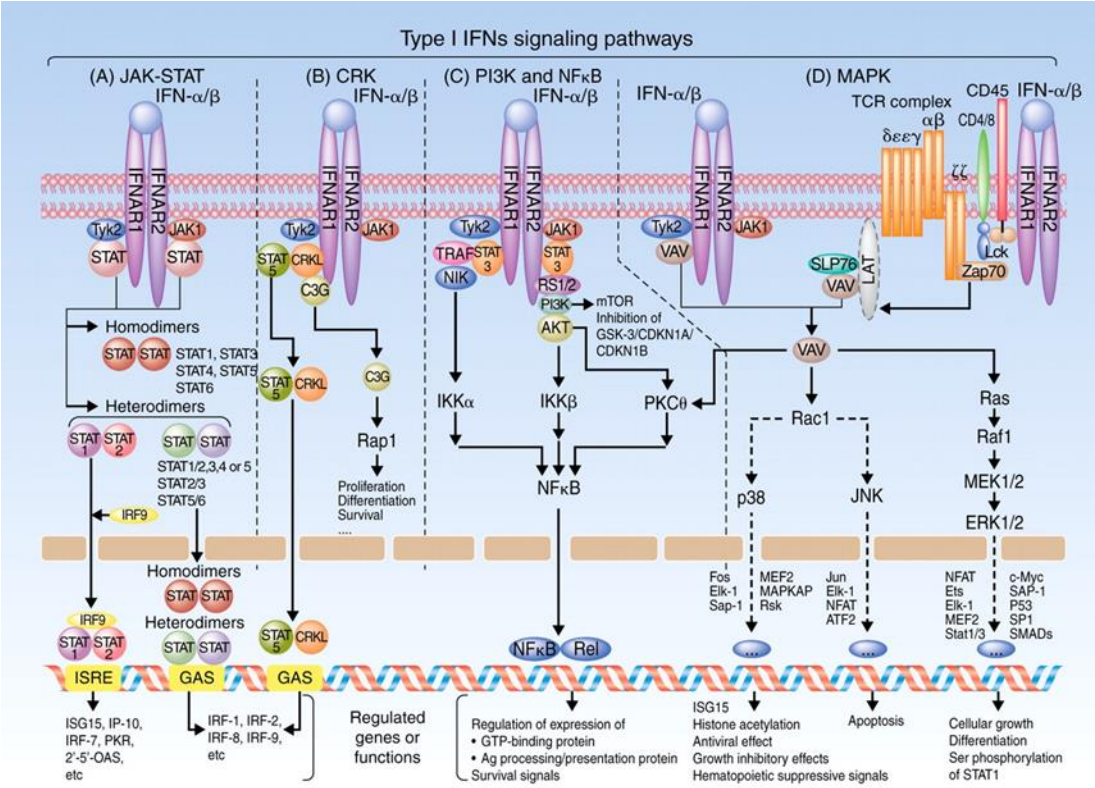

Figure 2.2. Type I IFN signaling pathways. Upon IFNAR engagement different signaling pathways including JAK-STAT, CRK, PI3K, NF-κB or MAPK pathway can be induced depending on the target cell and introduce a specific IFN gene expression profile. (Hervas-Stubbs et al., 2011)

2.1.3. General functions of type I IFNs

As a result of the ubiquitous expression of IFNAR and the numerous signaling pathways introduced upon receptor engagement, type I IFNs influence a broad variety of cellular processes. Micro array studies have identified a set of at least 1000 ISGs that were induced or repressed by type I IFNs. Besides inflammatory, anti-microbial and anti-viral ISGs also immunoregulatory ISGs, pro- and anti-apoptotic ISGs, as well as ISGs influencing proliferation and maturation of the target cells were identified (de Veer et al., 2001).

The first functional ability described for type I IFNs was their anti-viral capacity. Mechanistically the type I IFN mediated inhibition of viral replication depends on cell type and virus. In general, multiple steps of the viral life cycle, such as viral entry, transcription, initiation of translation, maturation, assembly and release are targeted by ISGs (Noppert et al. 2007). For example 2´-5´oligodeadenylate synthetase is an IFN dependent enzyme that can

induce the degradation of viral RNA (Zhou et al., 1993). Another type I IFN induced enzyme is protein kinase R (PKR). By the phosphorylation of different substrates PKR can block the translation of viral RNA (Lu et al., 1999). Furthermore, Mx proteins as typical anti-viral ISG products oligomerize around the viral nucleocapsid structure. Conformational changes upon GTP binding and/or hydrolysis then lead to disintegration of the infecting nucleocapsids (Gao et al., 2011; Haller et al., 2010).

Besides the anti-viral action type I IFNs like IFN-β strongly influence proliferation and maturation of certain cell subsets. On the one hand they repress myelopoiesis (Hwang et al., 1995) or proliferation of effector T cells (Petricoin et al., 1997) but on the other hand they may stimulate the survival of memory T cells (Sun et al., 2000). Moreover, type I IFNs are involved in cell cycle control by influencing c-myc expression (Hu et al., 2005), modulating cycline-dependent kinase (cdks) activity (Kumar and Atlas, 1992) and regulating the production of growth factors as well as their receptors (Noppert et al. 2007). In addition to this anti-proliferative action, type I IFNs influence the expression of several constituents of the intrinsic and extrinsic apoptosis pathways like BCL2 proteins, caspases or TNF family receptors and ligands. Therefore, they do not only exert cytostatic but also cytotoxic effects mediated via FADD/caspase 8 signaling (Chawla-Sarkar et al., 2003).

More recently, evidence accumulated indicating type I IFNs to be central regulators of innate and adaptive immune responses. An overview of the immunomodulatory effects assigned to type I IFNs is schematically depicted in figure 2.3.(Gonzalez-Navajas et al., 2012).

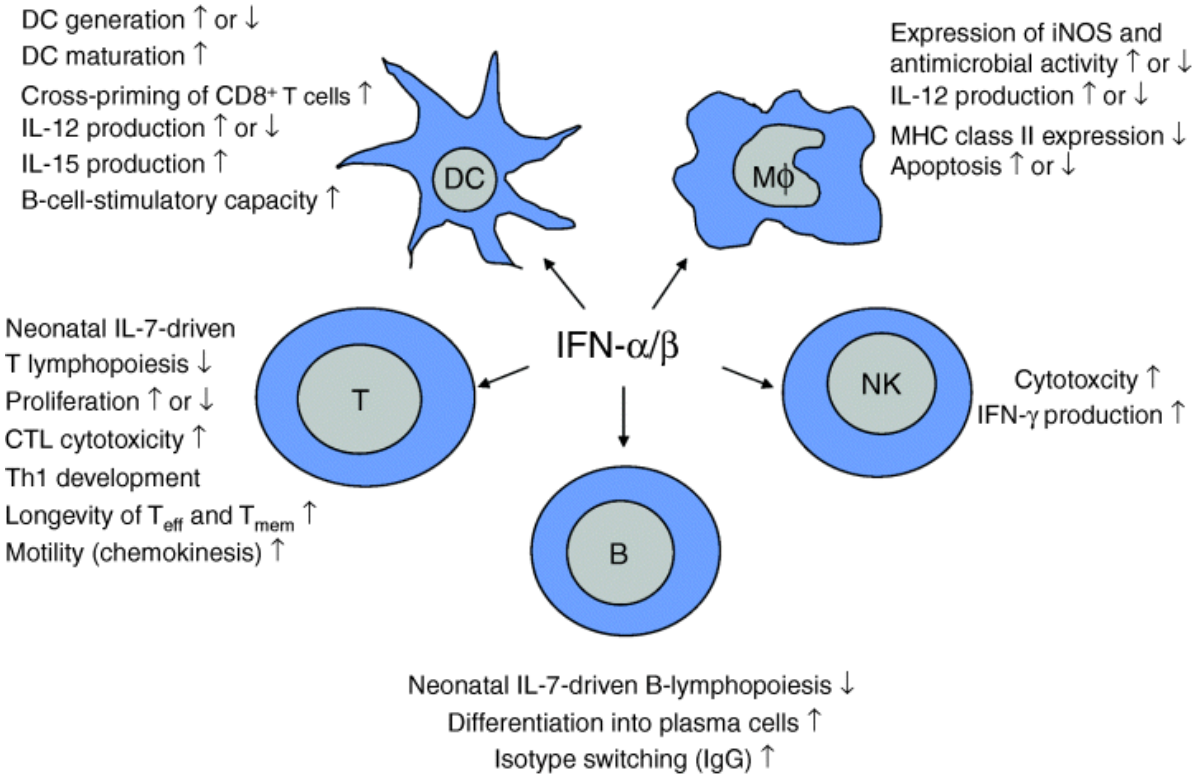

Figure 2.3. Immuno modulatory functions of type I IFNs. Type I IFNs influence several cellular components of the innate and adaptive immune system such as T cells, B cells, NK cells, DCs and macrophages (Bogdan et al., 2004)

Regarding the innate immune system, type IFNs have been reported to regulate NK cell functions like their killing ability, IFN-γ production or survival (Nguyen et al., 2002). Similarly, the monocyte and macrophage compartment is strongly influenced by type I IFNs. They seem to stimulate the antibody dependent cytotoxicity of macrophages, the differentiation of monocytes into DCs with high antigen presentation capabilities and they repress or induce the production of different macrophage derived cytokines (Bogdan et al., 2004).

Not only innate immune cells are affected by the presence of type I IFNs. With regard to the regulation of adaptive immune responses, type I IFNs have been shown to have effects on DCs, B cells and T cells. In addition to driving Th1 immune responses (Proietti et al., 2002), they can influence T cell proliferation and survival (Tough, 2012) and support CTL differentiation via the induction of T-bet expression (Wiesel et al., 2012). Besides cellular immunity, type I IFNs were demonstrated to play a crucial role for the development of humoral immune responses by supporting B cell survival and class switch (Kiefer et al.,

2012). In face of these potent effects of type I IFNs on the host immune system, a strong impact on cancer progression and development is a logical consequence.

2.1.4. The role of type I IFNs in tumor development

Shortly after the discovery of IFN by Isaacs and Lindenmann in 1957, the investigation of its role in tumor biology started. Nowadays, a strong anti-tumor effect of type I IFNs is well established and can be attributed to both, direct and indirect actions on tumor cells. It is known that type I IFN can directly induce growth arrest and apoptosis in certain tumor cells via the induction of pro-apoptotic genes like TRAIL and Fas or exert its cytostatic capacity via IRF9 (Bekisz et al., 2013). Additionally, IFN-β can upregulate the expression of the tumor suppressor p53 and inhibit transformation of mouse embryonic fibroblasts *in vitro* (Takaoka et al., 2003). Thus, the immunoregulatory potential of type I IFNs is of utmost importance for cancer biology. For instance, type I IFNs can enhance the expression of histocompatibility antigens, therefore allowing the recognition of tumor antigens that leads to the induction of an anti-tumor immune response (Wan et al., 2012). On the other hand, type I IFNs can stimulate certain subsets of immune cells that are important for cancer immunosurveillance or facilitate their recruitment (Hervas-Stubbs et al., 2011). Finally, type I IFNs play an important role in the process of cancer immunoediting, proving their significance during tumor development (Dunn et al., 2006).

Interestingly, many tumors show alterations in IFN response pathways. For example glioblastoma cells upregulate USP18, a negative regulator of IFN responses (Sgorbissa et al., 2011). Similarly, in melanoma patients genetic variants of type I IFN genes were demonstrated to exert a significant impact on survival and therapeutic response (Lenci et al., 2012), again highlighting the therapeutic potential of type I IFNs or their targets for cancer therapy.

2.1.5. Type I IFNs in cancer therapy

Intron-A (IFN-α2b, Schering-Plough) and Roferon-A (IFN-α2a, Hoffmann-La Roche) were the first FDA-licensed IFNs for tumor therapy. Initially, IFNs were used to treat hairy cell leukemia, but since then they were tested as therapeutic agents in a broad variety of cancers

(Fig. 2.4.) such as chronic myeloid leukemia, melanoma, lymphoma, HIV-associated Kaposi sarcoma or renal cell carcinoma (Wang et al., 2011).

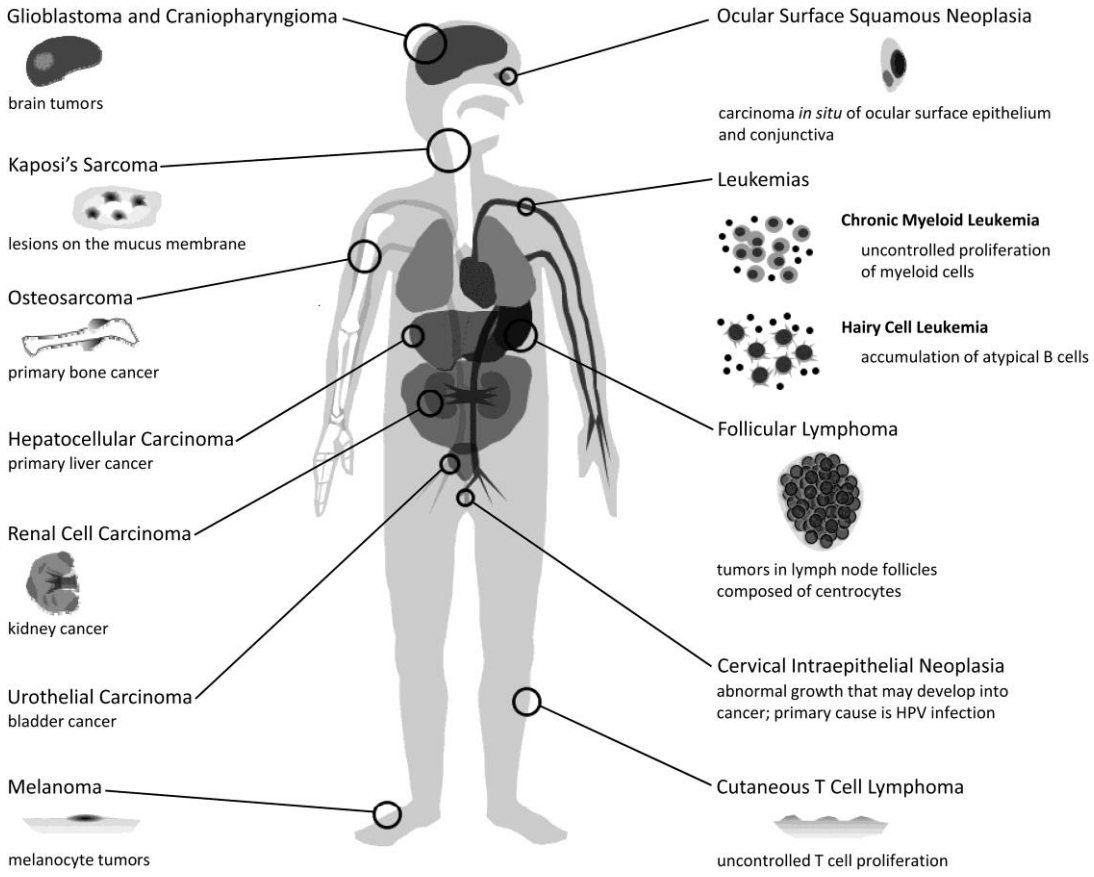

Figure 2.4. Human cancer targets of interferon therapy. IFNs are in clinical use for the treatment of a wide variety of solid and hematologic malignancies (Wang et al., 2011).

In patients suffering from high-risk cutaneous melanoma high-dose adjuvant IFN-α therapy improved both, relapse-free as well as overall survival (Mocellin et al., 2013). Also in the treatment of hematologic neoplasias such as chronic myeloid leukemia, polycythemia vera or hairy cell leukemia, IFN-α was demonstrated to induce remission and serve as a suitable maintenance therapy (Anguille et al., 2011). Interestingly, in CML patients IFN-α seems to activate cytotoxic T cells resulting in a long-term immunological control of neoplastic cells (Burchert et al., 2003).

Another tumor entity successfully treated with IFN-α, especially in combination with monoclonal antibodies against tumor antigens, are metastatic renal cell carcinoma. Of note, a combination of bevacizumab or sunitinib with IFN-α is the current standard of care for

advanced renal cell carcinoma in Germany (Melichar et al., 2008). Moreover, IFN-β in combination with temozolomid results in a favorable outcome for patients suffering from primary glioblastoma multiforme (Motomura et al., 2011). Taken together, type I IFNs alone or in combination with other drugs seem to exhibit an immunostimulatory effect and help to improve the therapeutic outcome of a wide range of solid and hematologic tumors (Wang et al., 2011). Therefore, understanding the molecular mechanisms targeted by IFN therapy could help to further improve their therapeutic use and potential.

2.2. Neutrophilic granulocytes

Neutrophilic granulocytes were first identified in the late 19th century by Paul Ehrlich who described them as polymorph nuclear cells with a tendency to retain neutral dyes. Evolutionary, neutrophils with functional similarity to mammalian neutrophils were present already in amphibians and bony fish. They play a major role in early immune responses and are central for host defense against bacterial pathogens (Amulic et al., 2012a). In contrast to humans, they are less abundant in other species.

2.2.1. Neutrophil development, life span and general functions

In the adult bone marrow, neutrophils differentiate from multipotent hematopoietic precursor cells called myeloblasts. During the maturation process, they pass different intermediate stages (Fig. 2.5) that can be characterized by the presence of different granules (Borregaard, 2010). These granules can be divided into three subsets (primary/azurophilic, secondary/specific and tertiary/gelatinase) each containing a specific composition of proteins, mainly enzymes, that are produced according to the genetic program active in the different neutrophil maturation stages (Liu et al., 2013). Azurophilic granules are largest in size and produced first during neutrophil maturation. Besides other anti-microbial proteins they contain myeloperoxidase (MPO), the main player in oxidative burst (Faurschou and Borregaard, 2003). The specific or secondary granules are smaller and can be characterized by the presence of lactoferrin and lysozyme. As the latter ones tertiary granules are MPO negative. They are mainly produced in metamyelocytes and band cells and comprise different metalloproteinases such as gelatinase (Faurschou and Borregaard, 2003). Besides the

different cargo, the release kinetics of these three granule subsets vary from difficult (azurophilic granules) to fast and easy (tertiary granules) (Borregaard and Cowland, 1997).

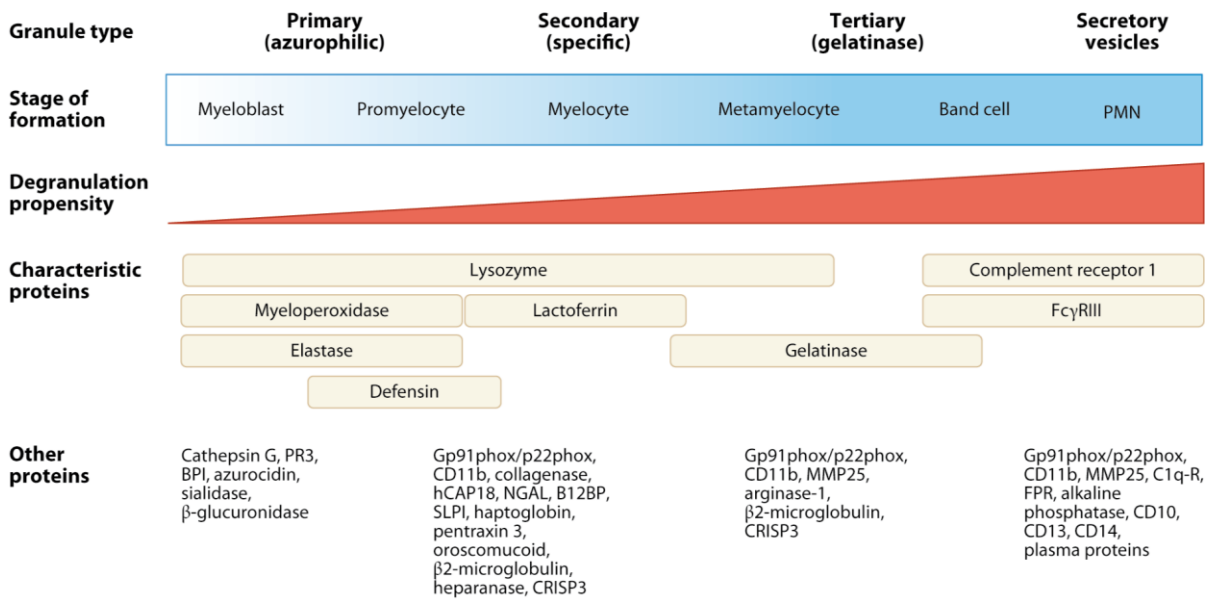

Figure 2.5. Neutrophil maturation and granule formation. The process of neutrophil maturation is accompanied by the formation of different granule subclasses each containing a specific composition of proteins (Amulic et al., 2012b).

In mice as well as in humans, neutrophil mobilization from bone marrow is tightly regulated by the expression of CXCR2 and CXCR4 chemokine receptors and their ligands. Meanwhile CXCL12 secreted by bone marrow stromal cells binds to CXCR4 and provides a key retention signal to neutrophils, the G-CSF induced CXC chemokines CXCL1 and CXCL2 signal via CXCR2 to initiate neutrophil mobilization (Eash et al., 2010). In general, the cycling time of neutrophils is comparably short. They tend to leave circulation after 6 to 8 hours upon the recognition of inflammatory sites intended by the presence of host- or pathogen-derived chemo attractants (Amulic et al., 2012a). Therefore, they bind to P-selectins, E-selectins and ICAMs on activated endothelial cells, extravasate into the target tissue and become activated during transmigration (Fig.2.6.).

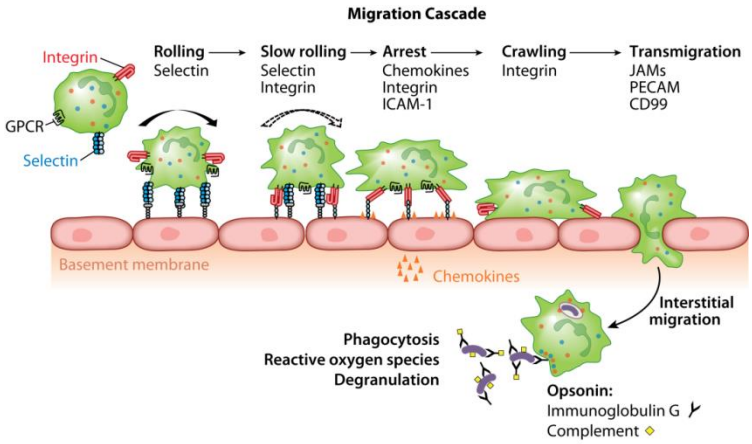

Figure 2.6. Migration and tissue infiltration of neutrophils. Trans-endothelial migration of neutrophilic granulocytes is a step-wise process facilitated by adhesion molecules present on activated endothelial cells and neutrophils (Mayadas et al., 2014)

Following gradients of chemoattractants such as fMLP, CXCL1, CXCL2 or IL-8 (only in humans) formation of the oxidative burst machinery is conducted. Besides the initiation of antimicrobial reactions, like receptor-mediated phagocytosis of pathogens, degranulation and the formation of neutrophil extracellular traps (NETs), neutrophil activation is accompanied by the secretion of pro-inflammatory cytokines crucial for the establishment of fully functional immune responses (Borregaard, 2010).

Therefore, patients suffering from genetic disorders or diseases associated with neutropenia display a frightening short survival. If treatment is lacking such patients die due to severe, non-resolving bacterial infections.

2.2.2. Neutrophils in immune regulation

Since neutrophils were long regarded as simple, terminally differentiated and short-lived effector cells, their immunoregulatory capacity has been largely underestimated. Nowadays, emerging evidence suggests that neutrophils have a strong impact on both innate and adaptive immunity, and cooperate with other immune cells in a complex cross talk as depicted in figure 2.7. (Mantovani et al., 2011).

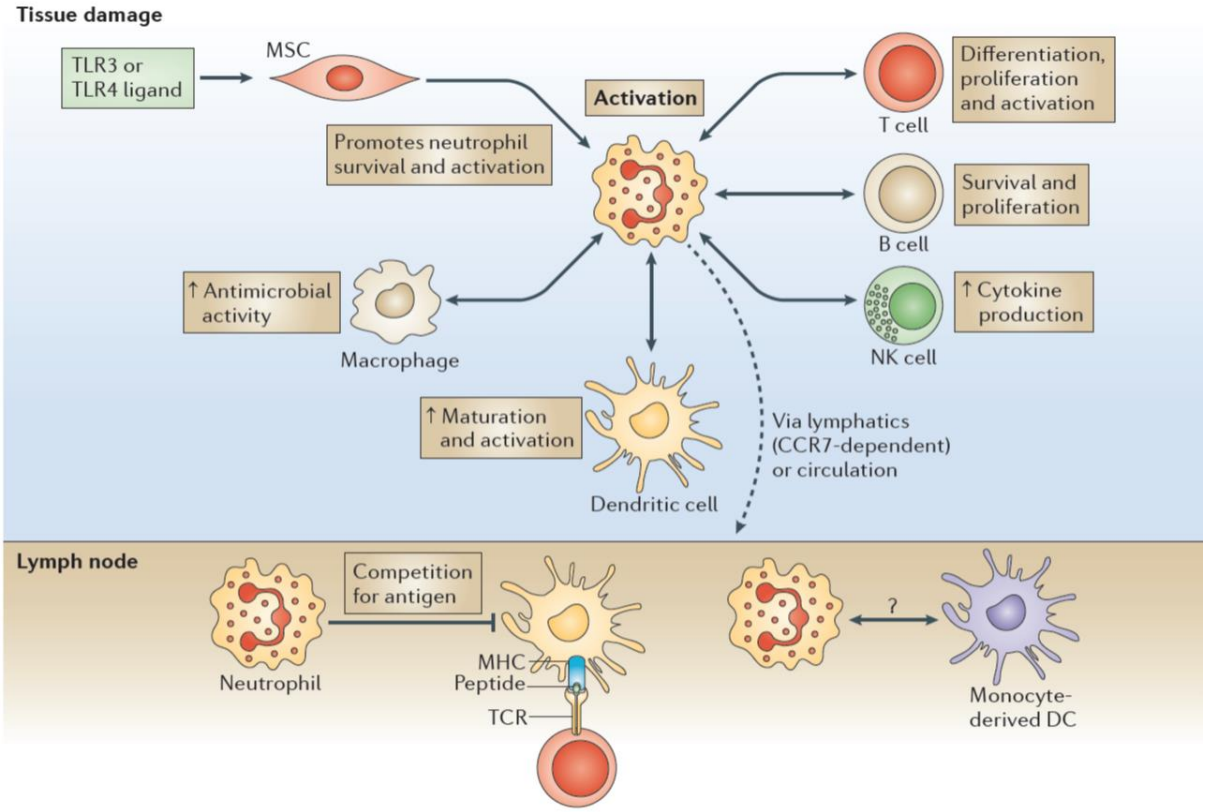

Figure 2.7. Neutrophil-immune cell cross talk in inflammation. During inflammation neutrophils undergo a bilateral crosstalk with macrophages, DCs, T cell, B cells and NK cells essential for a functional immune response (Mantovani et al., 2011).

Several neutrophil derived products such as lactoferrin, α-defensins or CCL3 were reported to have a chemotactic effect on other myeloid cells, such as DCs (Yang et al., 2009). In addition, direct contact with neutrophils can facilitate maturation of DCs and increase their antigen presentation efficacy (Alfaro et al., 2011). Neutrophil-DC interactions are not restricted to processes of host defense. For example in autoimmune diseases like SLE the release of NETs can be linked to disease exacerbation and the activation of DCs. Interestingly, the IFN-α secreted due to this interaction further enhances NET formation (Lande et al., 2011).

Another bilateral regulation is described for neutrophils and T cells. Neutrophils can act as APCs and express MHC class II to induce Th1 or Th17 polarization of $CD4^+$ T cells (Abi Abdallah et al., 2011). Interestingly, neutrophil antigen presentation is not restricted to the site of inflammation, but can also occur in lymph nodes and bone marrow (Beauvillain et al., 2007, 2011; Duffy et al., 2012). In addition to stimulatory effects neutrophils are known to

inhibit T cell proliferation via the secretion of arginase or reactive oxygen species (ROS) (Mayadas et al., 2010).

Besides their effects on DCs and T cells, B cells are also influenced by neutrophils. Their survival and activation is facilitated by neutrophil derived cytokines like BAFF (B cell activating factor) or APRIL (a proliferation-inducing ligand) (Scapini et al., 2008). In spleen neutrophils can indeed be found next to the marginal zone where they carry out B cell support and are termed B-cell helper neutrophils (N_{BH}) (Cerutti et al., 2013).

Recently, several interactions of neutrophils and NK cells have been elucidated indicating that neutrophils regulate NK cell maturation and function (Jaeger et al., 2012). On the other hand, activated NK cells produce neutrophil survival factors such as GM-CSF that initiate a positive feedback loop and augment inflammatory responses (Costantini et al., 2011).

Last but not least, neutrophils and macrophages interact and influence each other. Upon activation, neutrophils produce large amounts of monocyte/macrophage attracting chemokines like CCL2, CCL3, CCL19 or CCL20 that recruit these cells to the site of inflammation (Amulic et al., 2012b). Moreover, neutrophil granule components can increase the anti-microbial activity and support the phagocytic capacity of macrophages (Soehnlein et al., 2009). Of particular importance is the interaction of macrophages and neutrophils in the resolution phase of inflammation. Here, macrophages are responsible for the clearance of apoptotic neutrophils that in turn induces an M2 anti-inflammatory phenotype in macrophages and helps tissue repair (Filardy et al., 2010). Besides, neutrophils actively contribute to the resolution of inflammation by the production of anti-inflammatory lipid mediators, resolvins and protectins as well as by cytokine scavenging via different CC and CXC chemokine receptors (Mantovani et al., 2011).

2.2.3. Neutrophil cell death – apoptosis, NETosis and clearance

In contrast to cells of the adaptive immune system, neutrophils need no activation to acquire an effector phenotype. In accordance with their pleiotropic function, neutrophils are equipped with a large array of effector molecules that can potentially harm the host. Therefore, neutrophil cell death and clearance need to be tightly controlled (Witko-Sarsat et al., 2011). In general, the mode of neutrophil cell death is context dependent. Besides apoptosis, they can undergo necrosis, NETosis or autophagy (Kennedy and DeLeo, 2009).

Neutrophil apoptosis is known to play a critical role during the resolution of inflammation and is necessary to retain homeostasis (Geering and Simon, 2011). It can be induced by both, the extrinsic as well as the intrinsic apoptosis pathway (Fig. 2.8.) (Gabelloni et al., 2013).

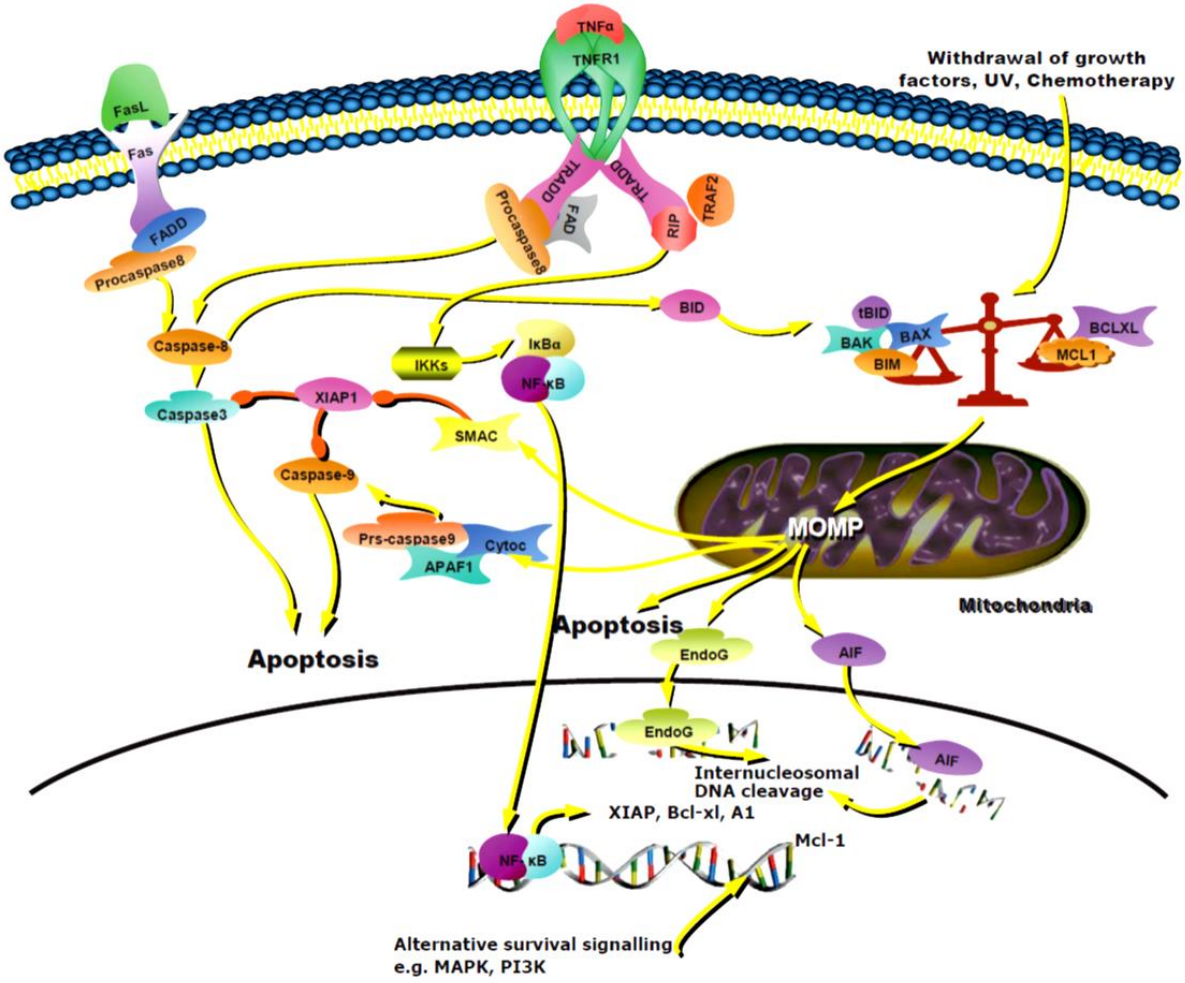

Figure 2.8. Neutrophil apoptosis pathways. Neutrophil apoptosis can be induced via the intrinsic and the extrinsic death receptor mediated pathway (Fox et al., 2010)

As a trigger for the extrinsic apoptosis pathway mainly macrophage released death receptor ligands such as Fas ligand or TNF-α are important. After binding to their receptors on the neutrophil cell surface they induce formation of the death-inducing signaling complex (DISC) that subsequently catalyzes the activation of initiator caspases 8 or 10. This finally leads to the induction of apoptosis conducted by effector caspase 3 (Galluzzi et al., 2012b). Neutrophils can be characterized as type II cells i.e. their extrinsic apoptosis pathway is mitochondria-dependent and they produce low amounts of active caspase 8. To this end,

neutrophilic death receptor signaling requires strengthening by an interplay with the intrinsic apoptosis pathway initiated by truncated Bid (Fox et al., 2010).

The intrinsic apoptosis pathway is responsible for the propagation of spontaneous neutrophil apoptosis. Here, mitochondria associated proteins of the BCL-2 family regulate mitochondrial outer-membrane permeabilization upon which cytochrome c is released into the cytoplasm. Subsequently, it induces apoptosome formation via Apaf-1 (apoptotic protease activating factor 1) oligomerization, leading to the activation of caspase 9. This initiator caspase in turn can cleave and activate effector caspases to induce cell death (Galluzzi et al., 2012b). Interestingly, the mitochondrial apoptosis pathway in neutrophils exhibits a surprisingly low threshold for cytochrome c that may be compensated by their comparably high expression level of Apaf-1 (Murphy et al., 2003). Concerning the occurrence of BCL-2 family members, neutrophils express the pro-apoptotic proteins Bax, Bak, Bad, Bid and Bik as well as the anti-apoptotic regulators Mcl-1 and BCL-xL (van Raam et al., 2006). On the pro-apoptotic side, especially Bax plays a non-redundant role in neutrophil apoptosis (Gardai et al., 2004). Remarkably, the neutrophil pro-survival factor G-CSF has been shown to down-regulate Bax expression (Dibbert et al., 1999). On the other hand, TNFα-induced neutrophil cell death has been demonstrated to be accompanied by an increased ratio of pro-apoptotic Bax towards anti-apoptotic BCL-xL (Perskvist et al., 2002).

Even though most of the mediators involved in neutrophil cell death are of general nature and involved in apoptosis of a broad variety of cell types, there are additional special features regarding neutrophils. For example ROS, that are produced in large amounts by activated neutrophils (Amulic et al., 2012a), seem to trigger neutrophil apoptosis via not yet fully understood mechanisms that involve an interplay with the classical pathway (Scheel-Toellner et al., 2004). Furthermore, NETosis, as a special form of neutrophil cell death, was described by Zychlinsky and colleagues (Brinkmann et al., 2004). During this process that is initiated by different pro-inflammatory stimuli such as LPS or TNFα, the neutrophil nuclear membrane is degraded and a homogenous mass of cytoplasm and nucleoplasm is released into the extracellular space. Such formations, called NETs are used to entrap and kill bacteria (Kaplan and Radic, 2012).

The major cells responsible for the clearance of apoptotic neutrophils are macrophages and DCs that sense signals exhibited by dying PMNs, e.g. lactoferrin, phosphatidylserine or

calreticulin (Bratton and Henson, 2011). This process, called efferocytosis plays a major role during the resolution of inflammation and for example induces the production of TGF-β or IL-10 by reprogrammed macrophages (Mantovani et al., 2011).

2.2.4. Neutrophils in tumor development and progression

Since Hanahan and Weinberg postulated the first six central hallmarks of cancer, namely, sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis (Hanahan and Weinberg, 2000), our understanding of the molecular mechanisms underlying this fatal disease has dramatically increased. The authors attributed to this by redefining a "next generation" of cancer hallmarks, that besides reprogramming energy metabolism and evading immune destruction, introduced two enabling characteristics i.e. genome instability and mutation as well as tumor-promoting inflammation (Hanahan and Weinberg, 2011). Indeed, the latter one is nowadays accepted to have a strong impact on most of the other hallmark capabilities highlighting the fact that a transformed cell alone is unable to establish a highly malignant and invasive lesion (Hanahan and Coussens, 2012). An overview of the interplay between tumor microenvironment and the hallmarks of cancer is depicted in figure 2.9.

Regarding the tumor-promoting effect of immune infiltration, mainly the innate arm of the invading immune cells has been claimed to be responsible. Besides macrophages, monocytes and immature myeloid cells (MDSCs), neutrophils play an imported role in this scenario (Galdiero et al., 2012). In the recent years, several clinical surveys have demonstrated the adverse prognostic effect of high neutrophil infiltration or blood neutrophilia in a broad variety of human cancers, including renal cell carcinoma, melanoma, glioblastoma or colorectal cancer (Donskov, 2013).

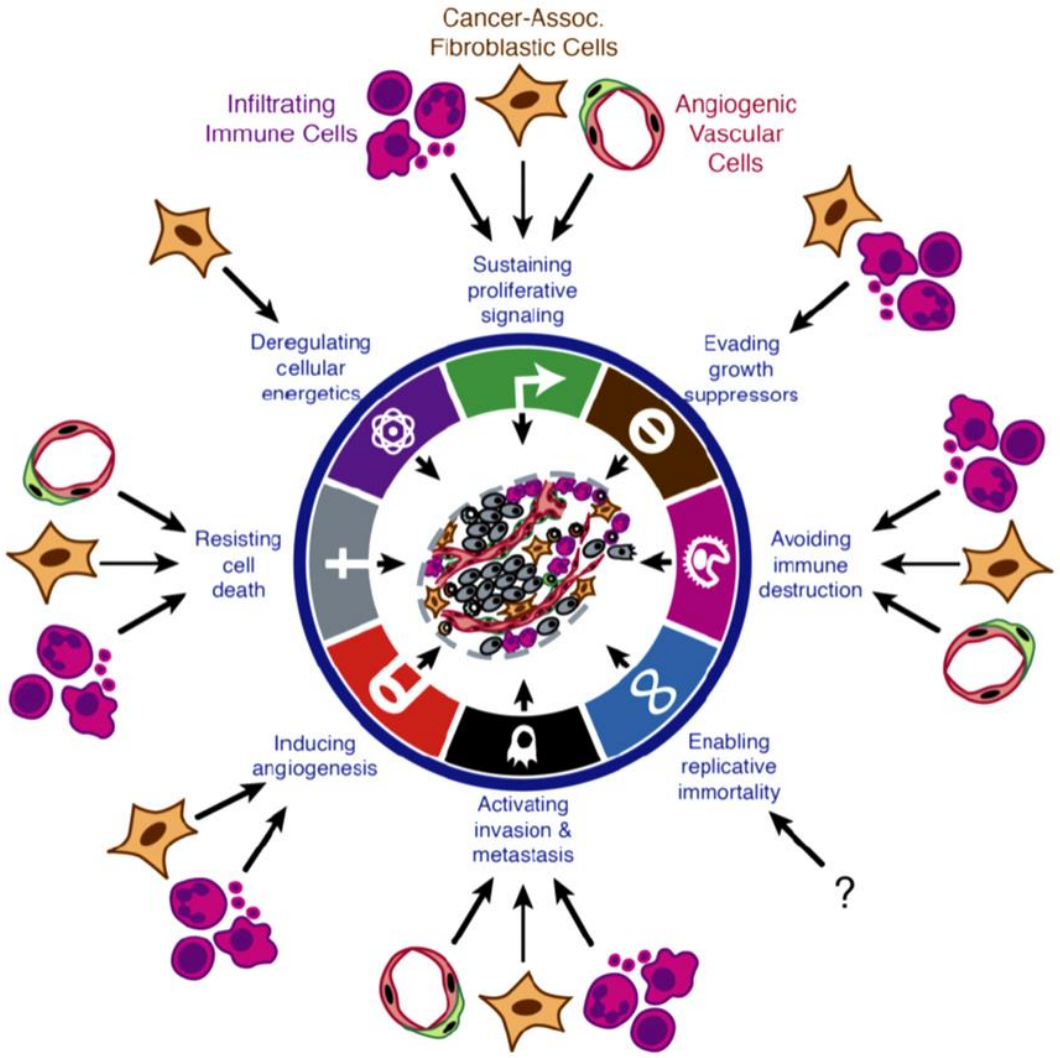

Figure 2.9. Influences of tumor stromal cells on the hallmarks of cancer. Tumor promoting inflammation is one of the most important hallmarks of cancer since invading immune cells influence almost all other cancer hallmarks (Hanahan and Coussens, 2012).

Indeed, neutrophils seem to be able to influence most of the critical hallmarks of cancer and promote massively the self-sustainment of a permissive tumor microenvironment via further facilitating the recruitment of leukocytes towards tumor site. This is due to the huge amounts of CXC chemokines like CXCL1, CXCL2, CXCL3 or CXCL5 secreted by the tumor associated neutrophils (TAN) (Jaillon et al., 2013). Moreover, general functions of neutrophils, such as the production of ROS could help to create a milieu that is permissive for genomic instability and mutation (Campregher et al., 2008). Similarly, the sustainment of

proliferative signaling can be promoted by TANs. As such, neutrophil elastase can cleave the insulin receptor substrate-1 (IRS-1), which in turn enhances PDGFR signaling and facilitates the proliferation of transformed cells (Houghton et al., 2010). The third hallmark of cancer influenced by neutrophils is the activation of tumor cell invasion and metastasis. To this end, TANs secret several factors that help tumor progression, such as oncostatin M (Queen et al., 2005) and hepatocyte growth factor (HGF) (Grenier et al., 2002).

In addition, TANs can promote angiogenesis, another hallmark of cancer, by expressing pro-angiogenic factors and matrix remodeling enzymes. Besides VEGF (Scapini et al., 2004), which is the major pro-angiogenic factor *in vivo*, they can secret Bv8 to induce the proliferation of endothelial cells (Shojaei et al., 2008). Moreover, TANs are the major source of MMP9, a pro-angiogenic matrix metalloproteinase (Kuang et al., 2011). Interestingly, matrix metalloproteinases can cleave cell-cell or cell-matrix adhesion molecules and thus block growth suppressing adhesion complexes (Hanahan and Coussens, 2012) or prevent tumor cell apoptosis (Acuff et al., 2006). Last but not least, neutrophils have strong immunomodulatory abilities. As already explained in chapter 2.2, they can modulate proliferation, maturation, recruitment, function and survival of a broad variety of immune cells including DCs, macrophages, monocytes, NK cell, B cells, effector T cells and regulatory T cells (Mantovani et al., 2011; Mishalian et al., 2014).. Under tumor conditions they can therefore help the tumor cells to avoid immune destruction for example by inhibiting T cell effector functions (Rodriguez et al., 2004) via the secretion of arginase (Rotondo et al., 2009)

2.2.5. Neutrophil polarization and plasticity– N1 anti-tumor vs. N2 pro-tumor phenotype

The micromilieu present in tumors provides a special niche and massively influences invading immune cells. This concept of immune cell polarization is well established for macrophages (M1/M2) under inflammatory conditions (Sica and Mantovani, 2012). M1 macrophages display a pro-inflammatory and anti-tumor phenotype and can be induced by IFN-γ alone or in combination with LPS, TNF-α or GM-CSF via STAT1 signaling (Schwartz and Svistelnik, 2012). These classically activated macrophages produce large amounts of IL-12, IL-23 and TNF-α and thus help to initiate Th1 and Th17 responses (Krausgruber et al.,

2011). Along this line in a tumor setting, M1 macrophages are capable to contribute to cancer immunosurveillance by orchestrating the T-cell-mediated tumor elimination (Schreiber et al., 2011). M2 macrophages on the other hand display an anti-inflammatory and pro-tumor phenotype. Alternative activation of macrophages as such is initiated by IL-4 and IL-13 via STAT6 signaling (Sica and Mantovani, 2012). In addition, IL-10 can promote an M2 pattern of gene expression in a STAT-3-dependent manner (Lang et al., 2002). Upon M2 polarization macrophages start to produce high amounts of IL-10 and arginase. Therefore, they are able to efficiently suppress Th1 responses and attenuate inflammation. Moreover, M2 macrophages are known to facilitate angiogenesis and tissue remodeling (Biswas and Mantovani, 2010). During tumor progression the M1/M2 ratio has been reported to shift towards an M2-biased direction (Zaynagetdinov et al., 2011).

In 2009 Fridlender and colleagues first described a phenotypic switch from pro- to anti-tumor functions for TANs. After TGF-β blockade, they observed a higher frequency of hypersegmented, cytotoxic and pro-inflammatory neutrophils in tumors of treated vs. untreated animals. These neutrophils were able to activate tumor-toxic $CD8^+$ T cells and seemed to be the target of TGF-β. Neutrophil depletion blunted the therapeutic effect of blocking agents (Fridlender et al., 2009). Since then, the investigation of TAN polarization gained increasing attention. It has been demonstrated that N2 TANs accumulate during tumor progression (Mishalian et al., 2013) and contribute to tumor growth (Pekarek et al., 1995; Tazawa et al., 2003) by a plethora of mechanisms described in the previous section and depicted in Figure 2.10 (2.2.4).

N1 neutrophils in contrast are tumor-toxic and inhibit tumor growth (Di Carlo et al., 2001; Hicks et al., 2006). Accordingly, depletion of neutrophils in cancer immunotherapies that are supposed to induce an N1 response abrogates therapeutic effects (Kousis et al., 2007; Suttmann et al., 2006). Along this line, N1 entrained neutrophils can inhibit tumor seeding in the pre-metastatic lung via the generation of H_2O_2 (Granot et al., 2011) or increase immune recognition of tumor cells via a neutrophil elastase dependent promotion of self-antigen presentation (Mittendorf et al., 2012). Moreover, direct killing of tumor cells by N1 neutrophils via the secretion of ROS (Zivkovic et al., 2007) or antibody-dependent cellular cytotoxicity (Hubert et al., 2011) has been demonstrated. Besides, N1 TAN can support an adaptive anti-tumor immune response. Therefore, they activate DCs via direct contact or the

secretion of TNF-α (van Gisbergen et al., 2005b; a) and modulate T cell responses under tumor conditions. For instance N1 neutrophils were demonstrated to recruit and activate T cells at tumor site by secreting chemokines like CXCL9 or CXCL10 or producing pro-inflammatory mediators like IL-12, TNF-α or GM-CSF (Fridlender et al., 2009; Scapini et al., 2000). Interestingly, antigen-pulsed neutrophils have also been shown to cross-present antigens *in vitro* to activate CTLs (Beauvillain et al., 2007). In addition, they help to activate $CD4^+$ T cells and establish an anti-tumoral T cell memory (Cavallo et al., 1992).

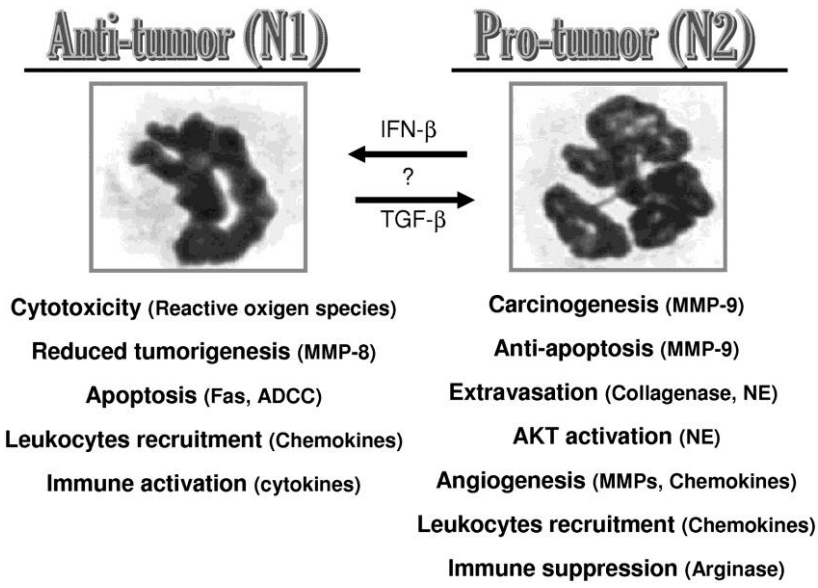

Figure 2.10. N1 vs. N2 polarization and functional properties of TAN. TGF-β and IFN-β are two potential factors influencing the functional polarization of TANs (Fridlender and Albelda, 2012).

Obviously, strict classification of TAN into N1 and N2 phenotypes is an oversimplification. More likely, these two immune phenotypes mark the extreme edges of a continuum of functional states that neutrophils can exhibit under tumor conditions (Mantovani et al., 2011). Still, it would be extremely promising to unravel the mechanism underlying polarization and alter the characteristics of tumor infiltrating neutrophils towards an N1 anti-tumor predominance. Currently, little is known about factors regulating the N1/N2 switch. On the one hand, TGF-β is suggested to be an N2 inducer (Fridlender and Albelda, 2012). Meanwhile data generated by Jablonska and colleagues point towards IFN-β as a potential N1 promoting cytokine (Jablonska et al., 2010). Nevertheless, neutrophils express also

receptors for other cytokines that could probably interfere with their polarization. For instance IL-10 as anti-inflammatory mediator (Moore et al., 2001), or IL-1β and TNF-α (Ferrante, 1992) could be involved in N1/N2 modulation. Thus, under tumor conditions IL-10 inhibits the secretion of pro-inflammatory IL-1β and TNF-α by TANs (Ferrante, 1992). Extrapolating these findings, the complex composition of pro- and anti-inflammatory mediators in the tumor microenvironment gradually shapes the immune phenotype of TAN and thus influences tumor promotion.

2.2.6. Neutrophils as type I IFN effector cells

Under certain inflammatory and infectious conditions neutrophils were identified as cells that produce type I IFNs and respond to them, retaining an effector phenotype. As such, neutrophils produce considerable amounts of IFN-α in the autoimmune disease systemic Lupus erythematosus (SLE) (Decker, 2011). In addition, type I IFNs have been demonstrated to induce the formation of NETs, further exacerbating SLE pathogenesis (Knight and Kaplan, 2012). Under infectious conditions neutrophil recruitment and activation seem to be influenced by type I IFNs. As consequence, in MyD88$^{-/-}$ animals with altered type I IFN responses neutrophil recruitment and activation were significantly reduced upon infection with *Burkholderia pseudomallei* leading to enhanced liver damage and death of the respective mice (Wiersinga et al., 2008). Moreover, in a model of septic shock, type I IFNs were shown to interfere with neutrophil recruitment and pharmacological inhibition of type I IFN signaling was able to block the onset of sepsis (Dejager et al., 2014)

Of note, type I IFNs also seem to exert important effects on neutrophils under tumor conditions. In absence of endogenous IFN-β, infiltration of neutrophils into the tumor was significantly enhanced thus resulting in notably faster tumor growth of transplantable MCA205 fibro sarcoma and B16F10 melanoma (Jablonska et al., 2013). Moreover, endogenous IFN-β seems to inhibit the expression of VEGF and MMP9 by neutrophils, thereby interfering with tumor angiogenesis and development (Jablonska et al., 2010). Interestingly, neutrophil accumulation and function seem to be controlled by endogenous IFN-β not only in the primary tumor. In fact, also the setup of a pre-metastatic niche in the lung of spontaneously metastasizing 4T1 mammary tumor bearing mice is boosted in the

absence of endogenous IFN-β independently of tumor size, thus facilitating a significantly faster formation of metastasis (Wu & Andzinski et al. 2014, submitted).

2.2.7. Coherency of TAN and myeloid derived suppressor cells

One property of an invasive malignancy is the establishment of a permissive microenvironment allowing progression and metastasis. As already explained in section 2.2.4 and 2.2.5., neutrophilic granulocytes are an important component in this scenario. Besides neutrophils, also myeloid precursor cells, the so called MDSCs, accumulate in the tumor bearing host and contribute to tumor progression (Gabrilovich et al., 2012a). MDSCs consist of a mixture of immature myeloid cells belonging to either the mononuclear (Mo-MDSCs) or polymorphonuclear (G-MDSCs) subgroup. The minimal immunophenotypic description of Mo-MDSCs is the co-expression of CD11b and Ly6C, meanwhile G-MDSCs can be characterized by surface expression of CD11b and Ly6G. Importantly, common feature of all MDSCs is their immunosuppressive functionality, carried out via different mechanisms including the production of arginase I (Brandau et al., 2013). Thus, MDSCs have been described to inhibit activation and proliferation of both $CD8^+$ as well as $CD4^+$ T cells (Bronte et al., 2000; Mazzoni et al., 2002). This process can be antigen-specific or unspecific (Nagaraj et al., 2007; Sinha et al., 2005). In addition, MDSCs were demonstrated to induce Tregs and M2 polarization of macrophages via the secretion of IL-10 and TGF-β (Sinha et al., 2007; Huang et al., 2006). Whether they also suppress NK cells is a matter of controversial discussion and most probably depends on the subset of MDSCs (Ostrand-Rosenberg and Sinha, 2009). Interestingly, MDSC accumulation seems not to be restricted to tumor situations, but can also be observed in response to bacterial infections (Delano et al., 2007), autoimmunity (Zhu et al., 2007) or stress (Jin et al., 2013; Kokolus et al., 2013). In general, the induction of MDSC accumulation is manly driven by pro-inflammatory mediators such as IL-1β or IL-6 (Bunt et al., 2007, 2006) and angiogenic factors like VEGF (Gabrilovich et al., 1998).

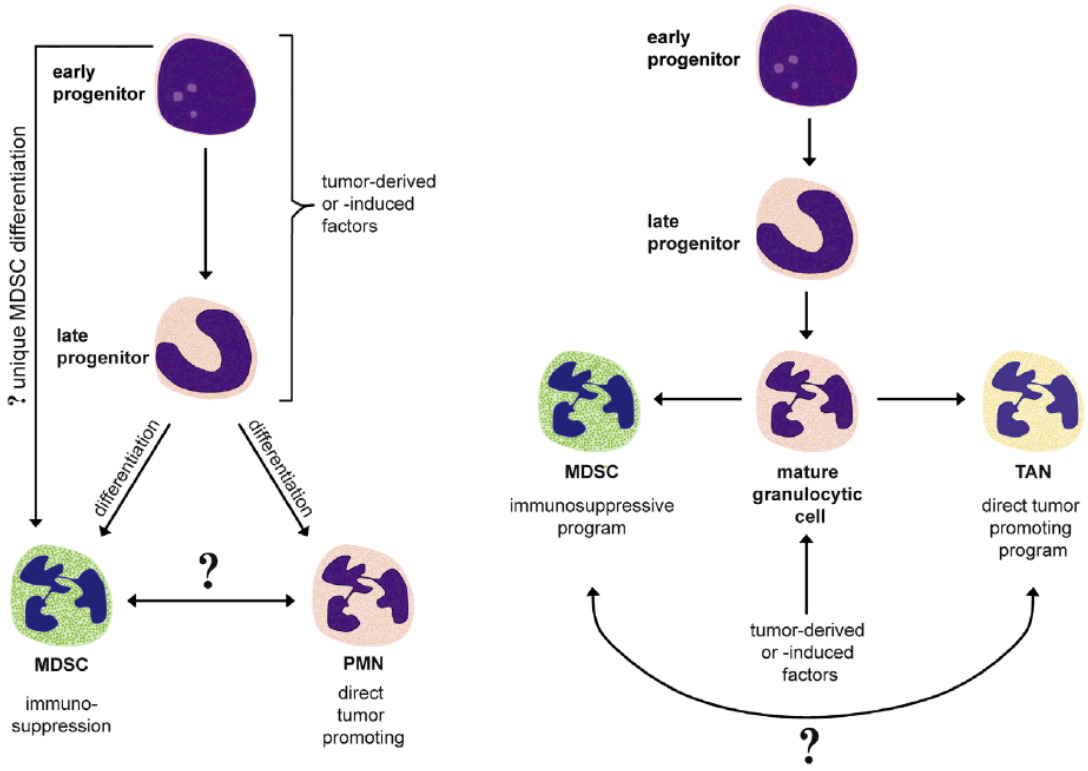

Figure 2.11. Potential differentiation pathways of TAN and granulocytic MDSCs. It is still under debate whether MDSCs differentiate from progenitor cells or mature granulocytes (Brandau et al., 2013).

To distinguish TAN and G-MDSCs is difficult. A clear cut definition of both cell subsets is currently missing. Indeed, there appears to be a significant overlap of TAN and G-MDSC functionality in the tumor-bearing host notwithstanding certain differences between the populations (Brandau et al., 2013). Just recently, large-scale gene expression analysis of G-MDSCs, naive neutrophils and TAN gave a first hint suggesting that G-MDSCs share more common features with naïve neutrophils than TAN. Nevertheless, in this study the different populations were isolated from different organs, clearly diminishing the impact of the reported findings (Fridlender et al., 2012). In the human system, only two studies report differences between G-MDSCs and TAN. In renal cell carcinoma, G-MDSCs secreted high amounts of arginase I while TAN did not (Rodriguez et al., 2009). In head and neck cancer, respectively, G-MDSCs showed decreased migratory capacities and LPS-induced IL-8 secretion (Brandau et al., 2011). However, the differentiation pathways of G-MDSCs vs. TAN (Fig.2.11) are elusive to date. While one theory suggests the independent development

of TAN and G-MDSCs from progenitor cells (Gabrilovich et al., 2012b), others describe G-MDSCs as neutrophil phenotype developing from mature granulocytes characterized by immunosuppressive functions. TAN on the other hand are characterized by direct tumor promoting capacity (Pillay et al., 2013). All facts considered, in this thesis TANs are defined as $Gr1^{hi}/Ly6G^{+}/Ly6C^{int}/CD11b^{+}/F4/80^{int}$ cells with a neutrophilic nuclear morphology and a lack of T cell suppressive functions.

2.2.8. TAN as target for tumor therapy

Priming of TANs towards a tumor-toxic N1 phenotype or depletion of N2 tumor-promoting neutrophils from the tumor bearing patients would be highly desirable aims for novel cancer immunotherapies. In general, two major strategies could be applied for this issue. On the one hand, targeting the CXCL8/CXCR2 axis could completely deplete TAN. However, also the N1 fraction would be reduced. On the other hand, certain TAN derived tumor-promoting factors could be targeted directly or TAN could be pharmacologically reprogrammed to develop anti-tumor functions (Gregory and Houghton, 2011). Indeed, concerning the interference with TAN migration, the current focus is on the development of receptor antagonists for CXCR2 (Walters et al., 2008). Another promising strategy is the specific inhibition of TAN derived neutrophil elastase that was shown to significantly retard tumor progression in mice (Houghton et al., 2010). Similarly, anti-angiogenic therapies that target neutrophil derived factors such as MMP9 are promising, especially in the light of the recently observed unfavorable outcomes of direct VEGF/VEGFR targeting (Quigley and Deryugina, 2012). Regarding neutrophil polarization into an N1 phenotype, the application of TGF-β blocking agents could be very efficient (Fridlender et al., 2009). In fact, this strategy has already reached the practical application as the TGF-β2 inhibitor AP12009 is in clinical trials for the treatment of glioblastoma (Hau et al., 2007). Moreover photodynamic therapy was demonstrated to induce the influx of immunostimulatory N1 neutrophils in mice (Kousis et al., 2007) and the efficacy of oncolytic viral infections seems to be boosted by the recruitment of neutrophils into tumors (Piccard et al., 2012). Additionally, neutrophils express the Fc receptors CD16 as well as CD32 and therefore display potential effector cells for antibody-dependent tumor cell lysis (Brandau et al., 2012). Interestingly, also the therapeutic effect of microbial compounds and biologics seems to be partly due to neutrophil

activation (Suttmann et al., 2006; Kandasamy et al., 2011). In summary, targeting TAN populations to improve cancer immunotherapy is a promising approach again highlighting the importance of further investigating TAN biology.

2.3. Aim of the work

Investigation of neutrophil polarization under tumor conditions and therapeutic intervention in this process gained increasing attention during the last years. Previous results suggest that the type I IFN family member IFN-β is of special importance regarding this issue. As described above, IFN-β was demonstrated to regulate neutrophil attraction (Jablonska et al., 2013) and pro-angiogenic capacity during tumor development, massively influencing tumor progression (Jablonska et al., 2010) and metastasis (Wu & Andzinski, 2014, under review). Therefore, it is of major interest to further elucidate the interplay of neutrophils and IFN-β in the tumor context, which was the central aim of this work. To this end, the nature of type I IFN signaling in tumors was characterized. More precisely, it was addressed whether constitutive IFN expression is responsible for neutrophil polarization at tumor site or whether IFN-β is induced by tumor or host derived factors. For this purpose, type I IFN induction in different transplantable tumor models was analyzed *in vivo* using an IFN-β reporter mouse approach. Furthermore, the potential pathways and cell types responsible for IFN-β induction and production were defined *in vivo* by the application of tissue specific conditional IFN-β reporter mice. Moreover, combinations of knock out mutants for different IFN induction pathways were used.

To generate a better understanding of IFN-β impact on the life span and polarization of TANs, detailed immunophenotypic characterization studies for N1/N2 TAN markers and their functional capacities were performed. In addition, turn over, maturation and apoptosis of neutrophils in tumor bearing IFN-β sufficient and deficient mice were assessed to create a broad overview of IFN-β mediated effects on neutrophilic granulocytes.

The effects of type I IFN tumor therapy are not fully understood to date. To this end the translational aspect of this work aimed to investigate the influence of type I IFN therapy on the neutrophil phenotype of mice using IFN-β treated tumor bearing wild type mice in comparison to untreated controls.

Taken together, the present work generates a deeper understanding of type I IFN-driven neutrophil polarization in the context of tumors. This should help to develop novel cancer immunotherapies targeting the neutrophil/type I IFN axis.

3. Material and Methods

3.1. Materials

3.1.1 Animals

All experiments performed in wild type animals were conducted using 8 to 12 week old female BALB/c (Harlan) or C57BL/6 (HZI breeding) mice. The transgenic animals used in the present work were bred in the animal facility of the Helmholtz Center for Infection Research (Braunschweig, Germany). Ifnb1$^{-/-}$, Rag2$^{-/-}$, Rag2$^{-/-}$xIfnb1$^{-/-}$, IFN-β$^{+/\Delta\beta\text{-luc}}$, IFN-β$^{+/flox}$ x Tie2 cre, IFN-β$^{+/flox}$ x LysM cre, IFN-β$^{+/flox}$ x CD11c cre, IFN-β$^{+/flox}$ x CD4 cre, IFN-β$^{+/flox}$ x CD19 cre or STING^{gt} mice employed in the present studies were backcrossed onto the C57BL/6 or BALB/c background for >15 generations All mice were kept under specific pathogen free (SPF) conditions. The studies have been reviewed and approved by an ethic committee of the regulatory authorities LAVES of Lower Saxony (33.9-42502-04-13/1122 permission number).

3.1.2 Cell lines

All tumor cell lines used in the present work were cultivated in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL; Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Integro), 250µmol/l β-mercaptoethanol (Serva), and 1% (v/v) penicillin/streptomycin (Sigma-Aldrich). Cells were grown in monolayer at 37°C in a humidified CO_2 incubator. B16F10 (C57BL/6), MCA205 (C57BL/6), LLC1 (C57BL/6), 4T1 (BALB/c) or CT26 (BALB/c) tumor cells were used in all experiments.

3.1.3 Antibodies

To perform flow cytometric analysis of single cell suspensions from bone marrow, blood, spleen, lung or tumor following antibodies were used:

Specificity	Clone	Fluorochrome	Company
CD16/32	2.4G2	none	BD Pharmingen
CD11b	M1/70	PE or APC	eBioscience
Gr1	RB6-8C5	PE-Cy7 or AlexaFluor 647	eBioscience
Ly6G	1A8	PE-Cy7 or AlexaFluor 647	Biolegend
Ly6C	AL-21	Alexa700	BD Pharmingen
Fas	15A7	PE	eBioscience
Active Caspase 3	C92-605	PE	BD Pharmingen
TNF-α	MP6-XT22	FITC	eBioscience
F4/80	BM8	PerCP-Cy5.5	eBioscience
ICAM1	YN1/1.7.4.	PE	eBioscience
CD45	30-F11	APC-Cy7	Biolegend
CD62L	MEL-14	APC	eBioscience
CD80	16-10A1	PE	eBioscience
CD86	GL1	FITC	BD Pharmingen
Arginase I		APC	R&D Systems

3.1.4 Primer

Real-Time RT-PCRs were performed with the following primers:

Bax: (s) 5´- ATG CGT CCA CCA AGA AGC TGA G-3´; (as) 5´- CCC CAG TTG AAG TTG CCA TCA G-3´

BCL-xL: (s) 5´- GGC TGG GAC ACT TTT GTG GAT-3´; (as) 5´- AAG CGC TCC TGG CCT TTC-3´

Caspase 3: (s) 5´- ATG GGA GCA AGT CAG TGG AC-3´; (as) 5´- TTG AGG TAG CTG CAC TGT GG-3´

mG-CSF: (s) 5´-CCA GAG GCG CAT GAA GCT AAT -3´; (as) 5´-CGG CCT CTC GTC CTG ACC AT-3´

Caspase 8: (s) 5´-TGC TTG GAC TAC ATC CCA CAC-3´; (as) 5´-TGC AGT CTA GGA AGT TGA CCA-3'

Apaf1: (s) 5'- CAG TAA TGG CGT CTT GTC AGT-3'; (as) 5´- AAG CGG CTG CTC GTT GAT ATT -3´

cFLIP: (s) 5´- GCT CCA GAA TGG GCG AAG TAA -3´; (as) 5´- ACG GAT GTG CGG AGG TAA AAA-3´

IFNb1: (s) 5´- CTG GCT TCC ATC ATG AAC AA-3´; (as) 5´- CAT TTC CGA ATG TTC GTC CT-3´

To standardize the cDNA the house keeping gene *Rps9* was tested with primer pairs:
(s) 5'-TTG ACG CTA GAC GAG AAG GAT-3'; (as) 5'-AAT CCA GCT TCA TCT TGC CCT-3'.

3.2 Methods

3.2.1 Murine tumor models

Exponentially growing tumor cells were harvested using 0.05% trypsin, washed, and suspended in LPS-free PBS. Tumors were initiated by subcutaneous (*s.c*) injection of $5x10^5$ cells in 100µl PBS. Tumor sizes were evaluated by caliper. All analytical experiments were done 12-14 days after initiation. The volume of a tumor was calculated using the formula: V = 4/3 * π * (h * w 2) / 8 (h = height and w = width). It was assumed that depth and width of the tumor are equal.

3.2.2 Low dose IFN-β therapy of tumor bearing mice

C57BL/6 or BALB/c mice were inoculated with B16F10 or 4T1 tumors. From day 3 after inoculation onwards they received i.v. treatment with 1000IU rmIFN-β every other day.

After two weeks the mice were sacrificed and single cell suspensions from blood, tumor and lung were analyzed by flow cytometry.

3.2.3 Flow cytometry of cells from tumor, lung, blood, bone marrow and spleen

To assess the immunological phenotype of neutrophil granulocytes from different tissues, single cell suspensions of blood, tumor, lung, spleen and bone marrow were analyzed by flow cytometry. Therefore, non-necrotic tumor tissue was cut into 1-2mm^3 pieces. The pieces were rinsed twice with PBS and digested using dispase/collagenaseA/DNase suspension in IMDM (0.2mg/ml 0.2mg/ml 100mg/ml) for 45min in 37°C. Cell suspensions were meshed through 50µm disposable filters (Cell Trics, Partec). Subsequently, erythrocytes were removed using erythrocyte lysis buffer (ACK buffer). To prepare single cell suspensions from lung tissue, the lungs were first perfused through the right heart chamber using 2 to 4ml cold PBS. Afterwards they were cut into small pieces, digested for 60min and treated as described above. Similarly, blood and bone marrow cell suspensions were treated with ACK buffer. To avoid unspecific binding of the conjugated antibodies single cell suspensions were treated with anti-mouse CD16/CD32 Fc block for ten minutes on ice. Then, cells were stained for 10 min in FACS buffer (PBS, 2%FCS, 0,5mM EDTA) with the conjugated antibodies listed above. To assess the expression of intracellular proteins the cells were subsequently fixed and permeabilized using BD^{TM} Cytofix-Cytoperm buffer for 20 min at room temperature in the dark. Afterwards, intracellular markers were stained with flurochrome-conjugated antibodies and flow cytometry was performed using the BD LSRII system (BD Bioscience). Data were analyzed by BD FACSDiva software (BD Bioscience).

3.2.4 Fluorescence activated cell sorting of neutrophils and DCs

Single cell suspensions from the different tissues were prepared and stained as described before. CD11b$^+$Gr1^{high} or CD11b$^+$Ly6G$^+$ neutrophils as well as CD11c$^+$CD11b$^+$ myeloid DCs or CD11c$^+$CD11b$^-$ conventional DCs were sorted using a FACSAria^{TM} cell sorter (BD Bioscience) and the purity of cells was proved by reanalysis.

3.2.5 BrdU labeling of bone marrow cells and FACS detection

To quantify neutrophil turnover, tumor bearing C57BL/6 as well as syngeneic *Ifnb1*$^{-/-}$ mice were challenged with B16F10 melanoma and i.v. injected with 1 mg BrdU on day 7 after tumor inoculation. 24, 48, 72, 96, 120, 144 and 168 hours later blood was taken from the mice via retro-bulbar punktion. After erythrocyte lysis (ACK buffer) the cells were stained for Ly6G and CD11b. Subsequently, BrdU was stained using BD BrdU Flow Kit (BD Bioscience) according to the manufacturers protocol and BrdU^{+} neutrophilic granulocytes were detected via flow cytometry.

3.2.6 Detection of reactive oxygen species via FACS

To detect the cellular ROS content, single cell suspensions of blood and tumor of C57BL/6 and syngeneic *Ifnb1*$^{-/-}$ mice were prepared as described above for flow cytometric analysis. Neutrophils were stained, resuspended in 200 µl RPMI ($\leq 1x10^6$ cells per well) containing 20 µM DCFDA and incubated for 1 hour at 37° in the dark. Subsequently, the cells were analyzed by flow cytometry without prior washing.

3.2.7 Flow cytometric analysis of cytochrome c

To detect mitochondrial cytochrome c release in apoptotic cells the InnoCyte^{TM} Flow Cytometric Cytochrome c Relaese Kit (Calbiochem) was used according to the manufacturer's protocol.

3.2.8 Detection of caspase 9 activity via flow cytometry

To analyze the activity of the initiator caspase 9 FAM-FLICA^{TM} Caspase Detection Kit (ImmonoChemistry Technologies) was used according to the manufacturers protocol.

3.2.9 *Ex vivo* apoptosis assay

To assess the *ex vivo* life span of neutrophil granulocytes derived from blood and tumors, single cell suspensions were stained as described before. CD11b^{+} Gr1^{+} neutrophils were

sorted and distributed in a concentration of $1x10^5$ cells per ml on a 96-well cell-culture dish. Cells were then incubated for 18 hours in RPMI containing 10% FCS at 37°C with or without the addition of 100 µM Apocyanin, 50 mM Taurine or 5 units/ml rmIFN-β. Subsequently, cells were stained with propidium iodid/AnnexinV and analyzed by flow cytometry.

3.2.10 *Ex vivo* tumor killing assay

To assess the *ex vivo* killing capacity of neutrophil granulocytes derived from blood and tumors, single cell suspensions were stained. $CD11b^+$ $Gr1^+$ neutrophils were sorted and distributed on a 96-well cell-culture dish. 20.000 neutrophils were co-cultivated overnight in RPMI (10% FCS, 1% (v/v) penicillin/streptomycin) with 10.000 Luciferase-expressing 4T1 cells to obtain an effector-target-ratio of 2:1. Subsequently, substrate solution (Luciferin Calipers) was added to each well and luciferase activity was directly measured in the IVIS 200 system (Calipers). The emitted light can be correlated to the number of living tumor cells per well.

3.2.11 T cell proliferation assay

25.000 $CD4^+CD25^-$ responder T cells (T conv) from wild type BALB/c mice were co-cultured in different ratios with either $CD4^+CD25^+$ regulator T cells (T reg) derived from wild type BALB/c mice, or sorted neutrophils from blood or tumors of wild type or *Ifnb1*$^{-/-}$ mice in the presence of 200.000 irradiated BALB/c spleenocytes. The cells were kept in 96 well round-bottom plates in IMDM + 10% FBS. T cells were stimulated with 1µg/ml soluble anti-CD3 (clone:145-2C11) for 72h at 37°C 5% CO_2. In the last 8-12h of culture proliferating T cells were pulsed with 1 µCi ^{3}H-thymidine to assess the proliferation rate.

3.2.12 *In vitro* treatment with rmIFN-β and RNA isolation

Sorted cells were cultured in microtiter plates at $1x10^4$ cells/well in 200µl RPMI and rmIFN-β was added at a concentration of 5 units/ml. After 4h of incubation, cells were harvested and total RNA was extracted using the RNeasy Kit (Qiagen) according to the manufacturer's protocol. DNA contamination was eliminated by incubation with DNaseI (Amersham

Pharmacia Biotech) and cDNA was prepared using RevertAid First Strand cDNA Synthesis Kit (Fermentas) and oligo(dT) as primers.

3.2.13 PCR and quantitative real time PCR (qRT-PCR)

To investigate the expression of genes relevant for neutrophil apoptosis or priming, qRT-PCR was performed using Power SYBR Green Master Mix (Applied Biosystems) and analyzed on a 7500 Real Time PCR System (Applied Biosystems).

PCR was performed with TrueStart *Taq*DNA Polymerase (Fermentas).

3.2.14 G-CSF detection via ELISA

Serum samples of tumor-bearing wild type and syngeneic *Ifnb1*$^{-/-}$ mice were collected on day 14 after tumor inoculation and kept at -80°C until the RayBio® Mouse G-CSF ELISA was used according to the manufacturer's description.

3.2.15 Western blot analysis

For preparation of cell extracts, tumor tissue was lysed using tissue homogenizer FastPrep-24 (MP) in buffer containing 250 mM Tris, 0.5% Triton X-100, and Halt protease inhibitor cocktail (Thermo Scientific). The following primary antibodies were used: anti-Phospho-PI3K p85 (Tyr458)/p55 (Tyr199) (Cell Signaling) and anti-Actin (MAB 1501R Chemicon). HRP–conjugated anti-rabbit and anti-mouse Abs (Amersham) were used as secondary Abs using ECL detection (Bio-Rad). The chemiluminescence signal was recorded digitally by a ChemiDoc DRS imaging system (Bio-Rad). Digital signal acquisition and analysis were performed using Quantity One Program, Version 4.6 (Bio-Rad).

3.2.16 Detection of luciferase activity *in* and *ex vivo*

For the determination of the enzymatic activity of luciferase, cells were lysed in Reporter Lysis Buffer (Promega). For luciferase activity assays from tissue, weight of tissue fragments was determined and fragments were homogenized in proportional volumes of Reporter Lysis

Buffer. Lysates were mixed with LARII (Promega) and measured in a luminometer. For *in vivo* imaging, mice were injected i.v. with 150 mg/kg of D-luciferin in PBS (Calipers), anesthetized using Isofluran (Baxter) and monitored using an IVIS 200 imaging system (Calipers). Photon flux was quantified using the Living Image 3.0 software (Calipers).

3.2.17 Cytospin analysis

To quantify the percentage of immature neutrophils derived from blood of tumor bearing C57BL/6 mice and syngeneic *Ifnb1*$^{-/-}$ animals, cytospin analysis of sorted Gr1^{+} CD11b^{+} neutrophils were performed. After centrifugation (10 min, 800 rpm, 4°C) of 100.000 cells on Superfrost™ slides (Thermo Scientific) they were dried and stained with Giemsa solution (Sigma Aldrich) for 15 min. Cells were fixed with ice cold methanol for 5 min and covered with Neomount® (Merck Millipore). Nuclear morphology was assessed by light microscopy (Zeiss) using 20x magnification. For each pooled sample at least 10 fields of view were counted and percentage of immature ring shaped nuclei was calculated from total neutrophils.

3.2.18 Statistics

Statistical analyses were performed using either unpaired students t test or one-way ANOVA in combination with Turkey post-test. Significances were defined according to a 95% confidence interval.

4. Results

4.1. Induction and production of IFN-β at tumor site

An influence of endogenous type I IFN signaling on growth and progression of different tumor entities has been described in mice and men (Jablonska et al., 2010; Swann et al., 2007; Deonarain et al., 2003; Picaud et al., 2002; Sgorbissa et al., 2011). Nevertheless, it is still unclear to date whether constitutively expressed low amounts of IFN-β (Gough et al., 2012; Lienenklaus et al., 2009; Taniguchi and Takaoka, 2001) or induced type I IFN responses are responsible for this phenomenon. Moreover, the type I IFN producing cell population(s) as well as potentially inducing ligands in the tumor are currently speculative (Fuertes et al. 2013). To this end, the present work addressed these issues *in vivo* using a reporter mouse approach.

4.1.1. IFN-β is induced in different transplantable tumor models on both BALB/c and C57BL/6 background

Type I IFNs such as IFN-β can be induced by a broad variety of factors under infectious as well as inflammatory conditions. These cytokines are produced and sensed by virtually all types of cells (Ivashkiv & Donlin 2013). Applying a previously described IFN-β reporter mouse model, which allows spatial and temporal resolution of IFN-β induction *in vivo* using firefly luciferase as reporter protein (Lienenklaus et al. 2009), the question of IFN-β induction during tumor development was addressed. To this end, 4T1 mammary carcinoma, CT26 colon carcinoma, MCA205 fibro sarcoma or LLC1 lung carcinoma cells were subcutaneously (s.c.) injected into the back of syngeneic IFN-β reporter mice, respectively. Imaging kinetics for a follow up period of 14 days were performed *in vivo* (Figure 4.1).

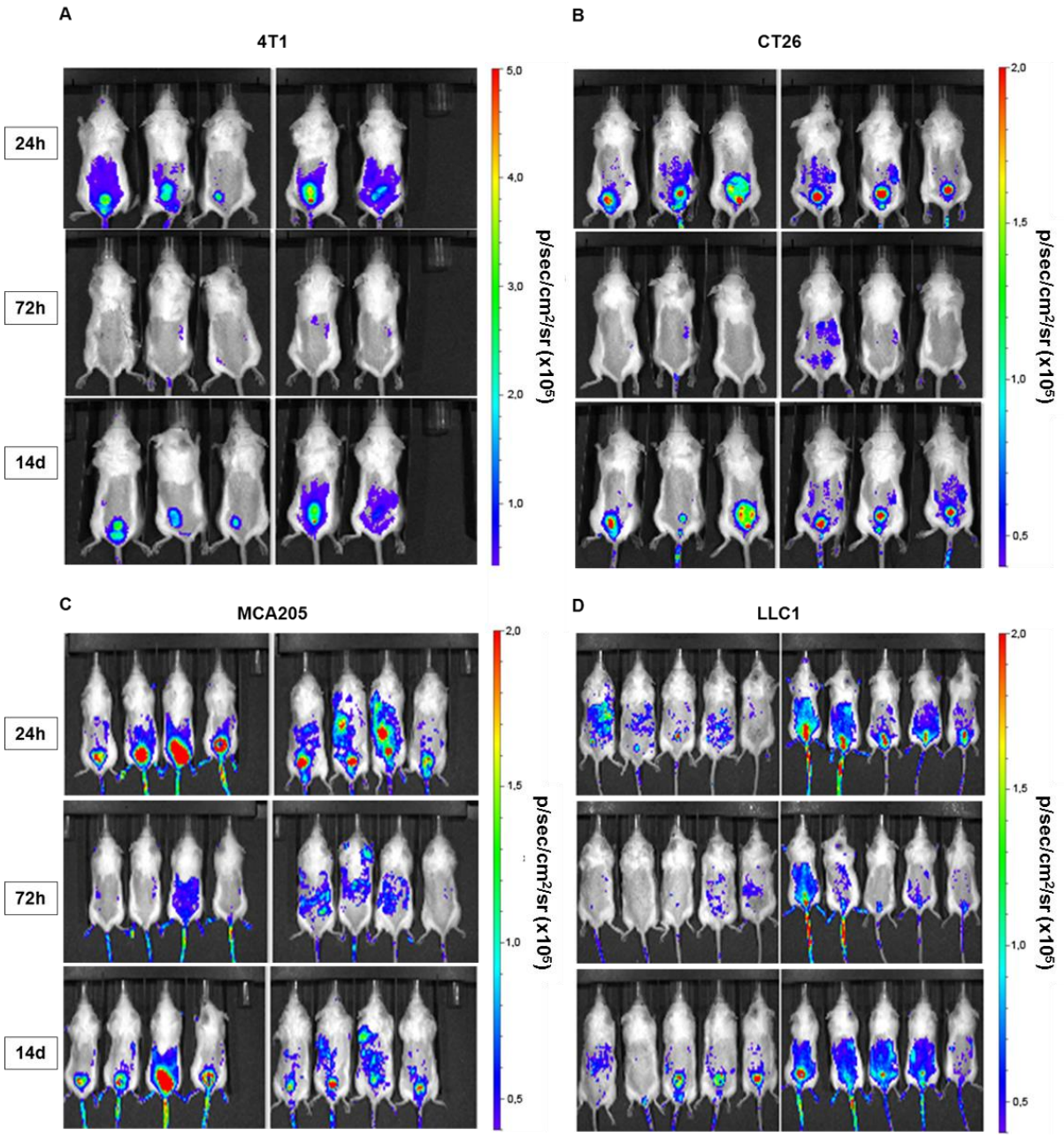

Figure 4.1. IFN-β induction in transplantable murine tumors. Transplantable 4T1 (A), CT26 (B), MCA205 (C) or LLC1 (D) tumor cells were s.c. injected into the back of IFN-β reporter mice on BALB/c or C57BL/6 background, respectively. Reporter activity was assessed after 24 hours and subsequently every other day, using an IVIS 200 imaging system. Depicted are the results obtained 24 hours, 72 hours and 14 days after tumor inoculation.

24 h after tumor injection an initial signal was detected at the inoculation site. This was true for all tested tumor models on both, BALB/c as well as C57BL/6 background. Expectedly, the signal was absent in PBS-injected control mice (Data not shown). By 72 hours after cell

injection, the signal was around constitutive background levels. At that time, no visible tumor could be found for any of the different tumor models. As soon as the tumor started to be palpable, the signal reappeared. On day 14 after inoculation the tumors were well established and the reporter signal reached levels observed 24 hours after injection indicating a type I IFN response in the growing tumors.

To verify and quantify the IFN-β induction in the different tumor models, reporter mice were challenged with MAC205 or CT26 tumor cells, respectively. On day 14 after tumor inoculation, the mice were sacrificed and tissue homogenates of tumor, spleen and thymus were tested for their *ex vivo* luciferase enzymatic activity indicating IFN-β induction (Fig. 4.2.).

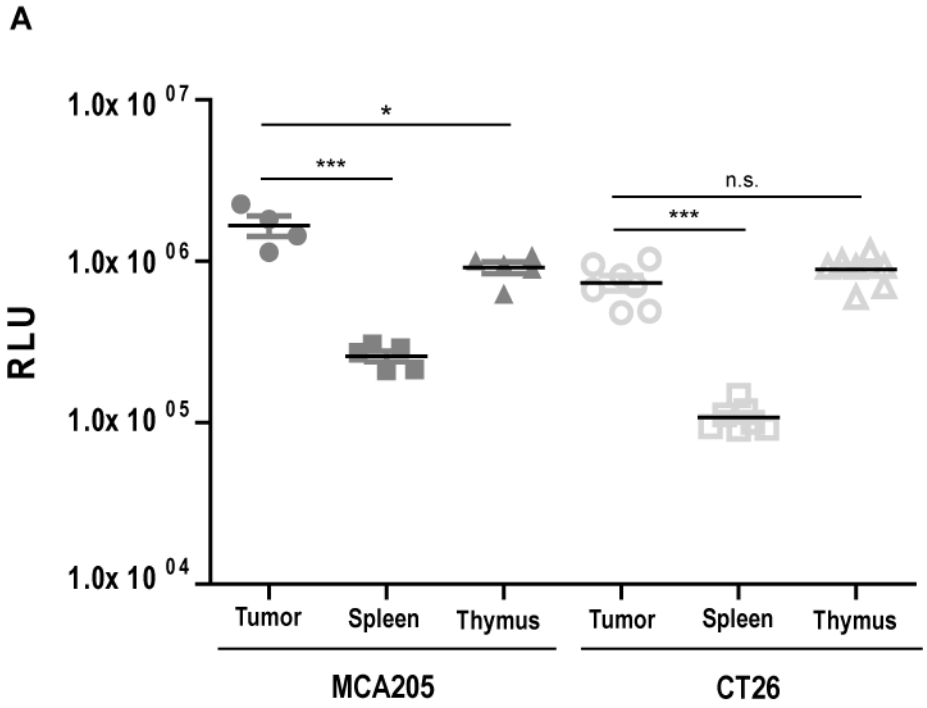

Figure 4.2. Ex vivo luciferase assay comparing IFN-β induction in different tumor models and different organs. On day 14 after s.c. inoculation of MCA205 or CT26 tumor cells into the back of IFN-β reporter mice on C57BL/6 or BALB/c background, reporter activity was assessed *ex vivo* using a luminometer to detect luciferase mediated bio luminescence in homogenates from tumor tissue, spleen or thymus. The relative luciferase units (RLU) were normalized on tissue weight.

Indeed, the luciferase signal detected in the tumor samples of MCA205 and CT26 tumors was significantly higher, compared to spleen, and reached at least the intensity of the thymic

IFN-β reporter activity. It was demonstrated previously that under steady state conditions, the thymus displays the highest constitutive IFN-β expression (Lienenklaus et al. 2009). In subsequent experiments, MCA205 was used as tumor model system since it induces high levels of IFN and is of appropriate genetic background.

4.1.2. Tie2$^+$ LysM$^+$ CD11c$^+$ DCs produce IFN-β at tumor site

DCs are the major source of type I IFNs in many settings. They have been demonstrated to produce IFN-β in tumor draining lymph nodes where they are involved in cross-priming of tumor antigen-specific CD8$^+$ T cells (Fuertes et al. 2011). Since it is not known whether DCs are also responsible for type I IFN production directly in the tumor, the present work tried to define the cellular source of the induced IFN-β in MCA205 fibro sarcoma. In a first experiment, the contribution of host cells and transplanted tumor cells was addressed. Therefore, C57BL/6 and syngeneic IFN-β$^{flox/flox}$ mice were inoculated s.c. with MCA205 cells. On day 14 after tumor injection the mice were sacrificed and RNA from total tumor tissue was isolated. After reverse transcription, PCR was performed to analyze IFN-β gene expression. In wild type mice as well as in tumor cells, the respective PCR product is 183 bp in size. IFN-β$^{flox/flox}$ mice (BLLB6) carry a loxP site in the 5'-UTR of the IFN-β locus. Therefore, the resulting PCR product has a size of 221 bp allowing to distinguish the signal derived from host vs. tumor cells. As depicted in figure 4.3. (A), both, host as well as tumor cells express IFN-β *in vivo*. Interestingly, MCA205 tumor cells did not produce detectable amounts of IFN-β in cell culture (Fig. 4.3. B). As control, also *Ifnb1* expression in total MCA205 tumor tissue was analyzed by qRT-PCR *ex vivo* (Fig. 4.3. B.). Importantly, this sample also contains host cells and does therefore not allow to distinguish the respective signal source.

To further characterize the host cell population responsible for IFN-β production at tumor site, specific conditional reporter mice were used. In these mice, the IFN-β coding sequence is replaced by the reporter luciferase upon cell specific Cre recombinase expression (Lienenklaus et al. 2009; Solodova et al. 2011). In the present work, Tie2Cre, CD19Cre, CD4Cre, LysMCre and CD11cCre were applied to detect IFN-β production from hematopoietic and endothelial cells, B cells, T cells, myeloid cells or DCs. The results

obtained from *ex vivo* luciferase measurements on day 14 after tumor inoculation are depicted in figure 4.4.

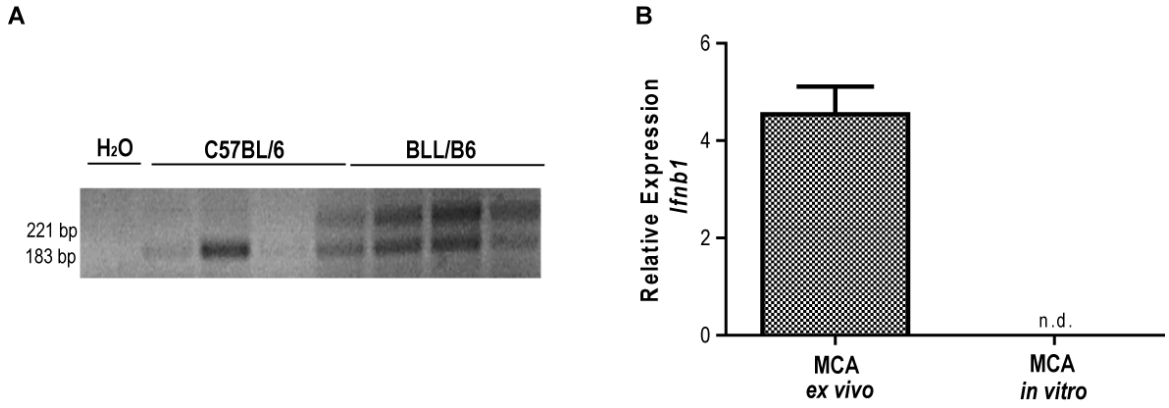

Figure 4.3. Host and tumor cells produce IFN-β in vivo. On day 14 after s.c. inoculation of MCA205 fibro sarcoma into C57BL/6 and syngeneic BLL/B6 (IFN-$\beta^{flox/flox}$) animals, total RNA from tumor tissue was harvested. After reverse transcription PCR analysis of IFN-β gene expression was performed (A) to distinguish host and tumor cell expression of IFN-β. In addition, qRT-PCR analysis of IFN-β gene expression were performed using MCA205 cells from cell culture (B).

For tumor homogenates of Tie2Cre, LysMCre and CD11cCre conditional IFN-β reporter mice, 100% equivalent signal intensity to the global reporter animals could be detected (Fig. 4.4. A to C). This indicates that first of all a cell population derived from hematopoietic precursor cells expressing Tie2 is responsible for IFN-β production at tumor site. In addition, the producing cells apparently express the myeloid lineage marker LysM and the integrin CD11c or expressed them during their ontogeny. Moreover, a subpopulation (~2%) of tumor associated host cells producing IFN-β in murine fibro sarcomas has expressed or still expresses CD4 (Fig. 4.4.D). Of note, no signal could be detected in CD19Cre reporter mice (Fig. 4.4.E), indicating that tumor infiltrating B cells do not contribute to IFN-β production in this experimental setting.

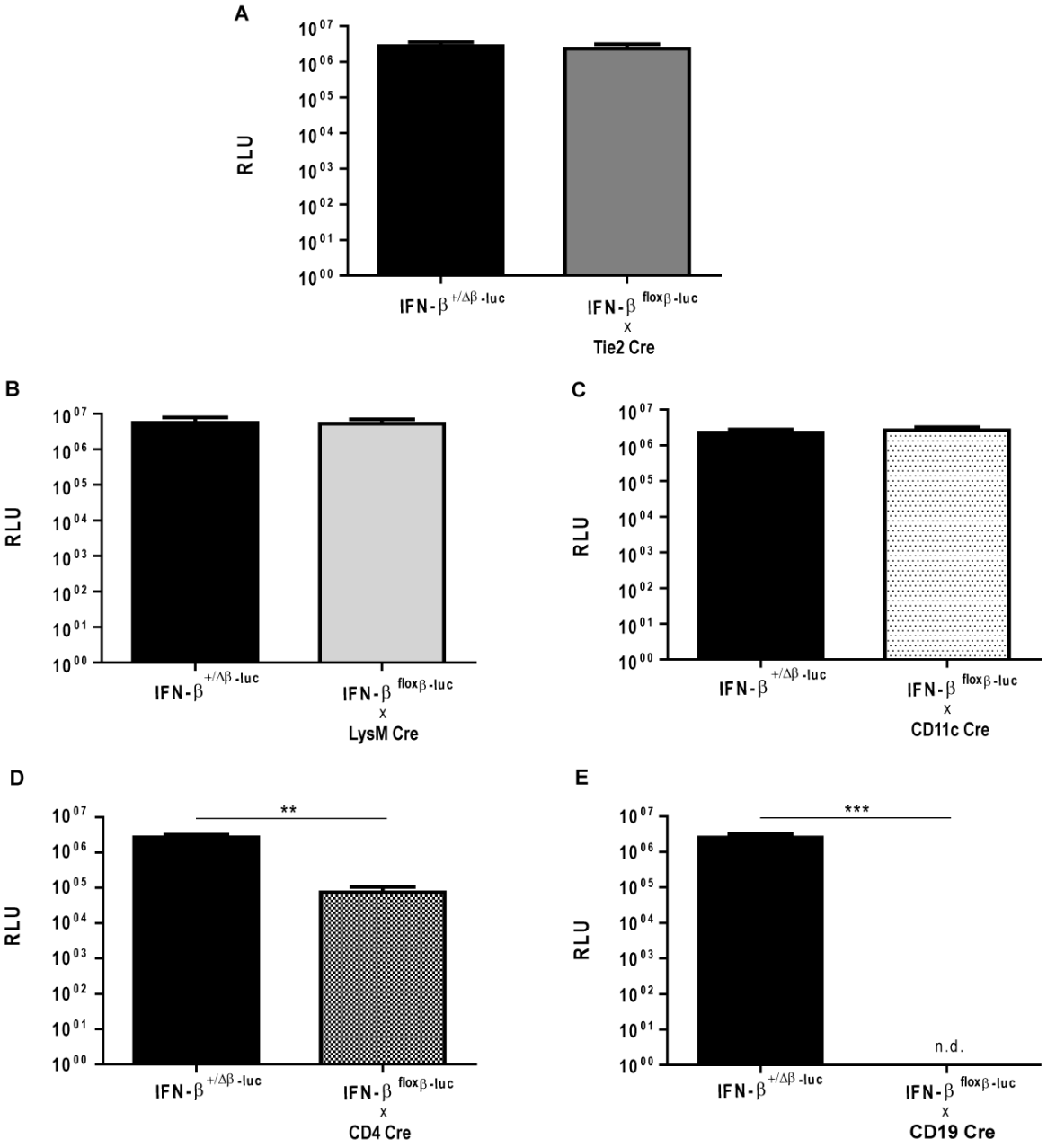

Figure 4.4. Phenotypic characterization of IFN-β producing tumor associated host cells. To assess the cellular source of IFN-β in tumors, IFN-β reporter mice as well as conditional Tie2Cre (A), LysMCre (B), CD11cCre (C), CD4Cre (D) and CD19Cre (E) reporter animals were injected with MCA205 cells. After 14 days the mice were sacrificed and ex *vivo* luciferase measurement was performed. The experiments were repeated at least once and statistical significance was calculated using unpaired students t test (** $p \leq 0{,}005$, *** $p \leq 0{,}0005$).

As murine DCs belong to the hematopoietic compartment, can be derived from myeloid precursor cells and express CD11c, they very likely are the cellular source of type I IFN under tumor conditions.

To validate this hypothesis, DCs populations from spleen and tumor of C57BL/6 mice were sorted and tested for gene expression of IFN-β (Fig.4.5.). By using splenic DC populations and $CD19^+$ B cells as control, it could be demonstrated that tumor associated DC populations express comparably high amounts of IFN-β. Thus, the data presented here provide strong evidence that myeloid dendritic cells are the major cellular source of IFN-β in solid tumors.

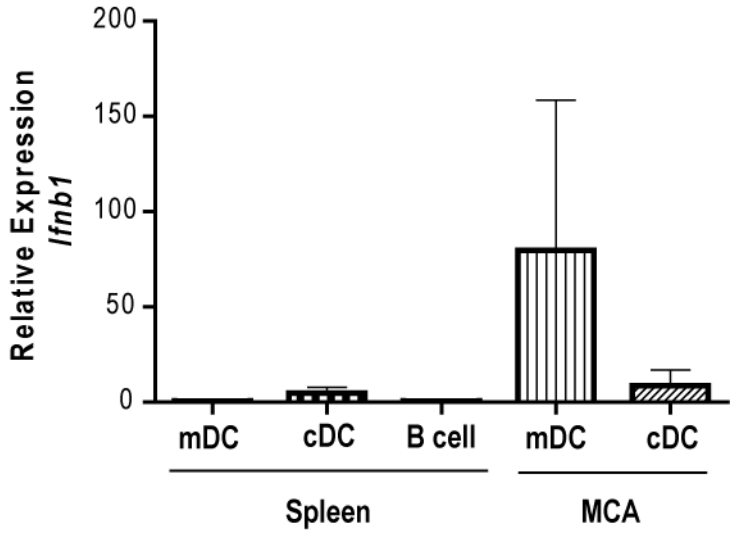

Figure 4.5. Tumor infiltrating DCs express IFN-β. To validate tumor infiltrating DCs as cellular source of IFN-β in the tumor microenvironment C57BL/6 mice were injected s.c. with MCA205 fibro sarcoma cells. On day 14 after tumor inoculation the mice were sacrificed and single cell suspensions from spleen and tumor prepared. $CD11c^+/CD11b^-$ cDC, $CD11c^+/CD11b^+$ mDCs and $CD19^+$ B cells were sorted using a BD FACS ARIA SORP system, RNA was isolated and qRT-PCR analysis for IFN-β gene expression were performed. Data represent a mean of at least five pooled animals. The experiment was repeated once.

4.1.3. Sting signaling is responsible for IFN-β induction in solid tumors

Three different receptor systems are known to induce type I IFNs upon ligand engagement. TLRs sense a broad spectrum of PAMPs and DAMPs and signal via the adaptor proteins Trif and MyD88 (Noppert et al. 2007). RLRs are cytosolic RNA receptors that signal via the adaptor protein Cardif/IPS-1/MAVS to induce type I IFN expression (Loo & Gale 2011) and the third receptor system capable to mount type I IFN responses are CDRs. They sense cytosolic viral, bacterial or host derived DNA and signal via the mitochondrial resident signal transducer Sting (Paludan & Bowie 2013). After integration of all signaling pathways by the central kinase TBK-1 type I IFN gene expression is induced by transcription factors of the IRF family, namely IRF3, IRF7 or IRF5, alone or in combination (Barnes et al. 2002).

Regarding IFN induction in the tumor microenvironment, neither the pathways nor the ligands responsible for type I IFN induction are identified to date (Fuertes et al. 2013). Therefore, the present work aimed to characterize the signaling pathways responsible at tumor site. To this end, IFN-β reporter mice were bred with mice carrying knockout mutations for molecules known to be involved in type I IFN induction. These mice were analyzed for their ability to induce IFN-β associated with the transplanted tumor. (Fig. 4.6.).

As demonstrated in Fig. 4.6 neither TLRs (Fig.4.6. A) nor RLRs (Fig.4.6. B) are involved in the induction of a type I IFN response in tumors. However, the lack of the adaptor protein Sting drastically decreased IFN-β gene expression at the tumor site in comparison to wild type animals (Fig.4.6. C).

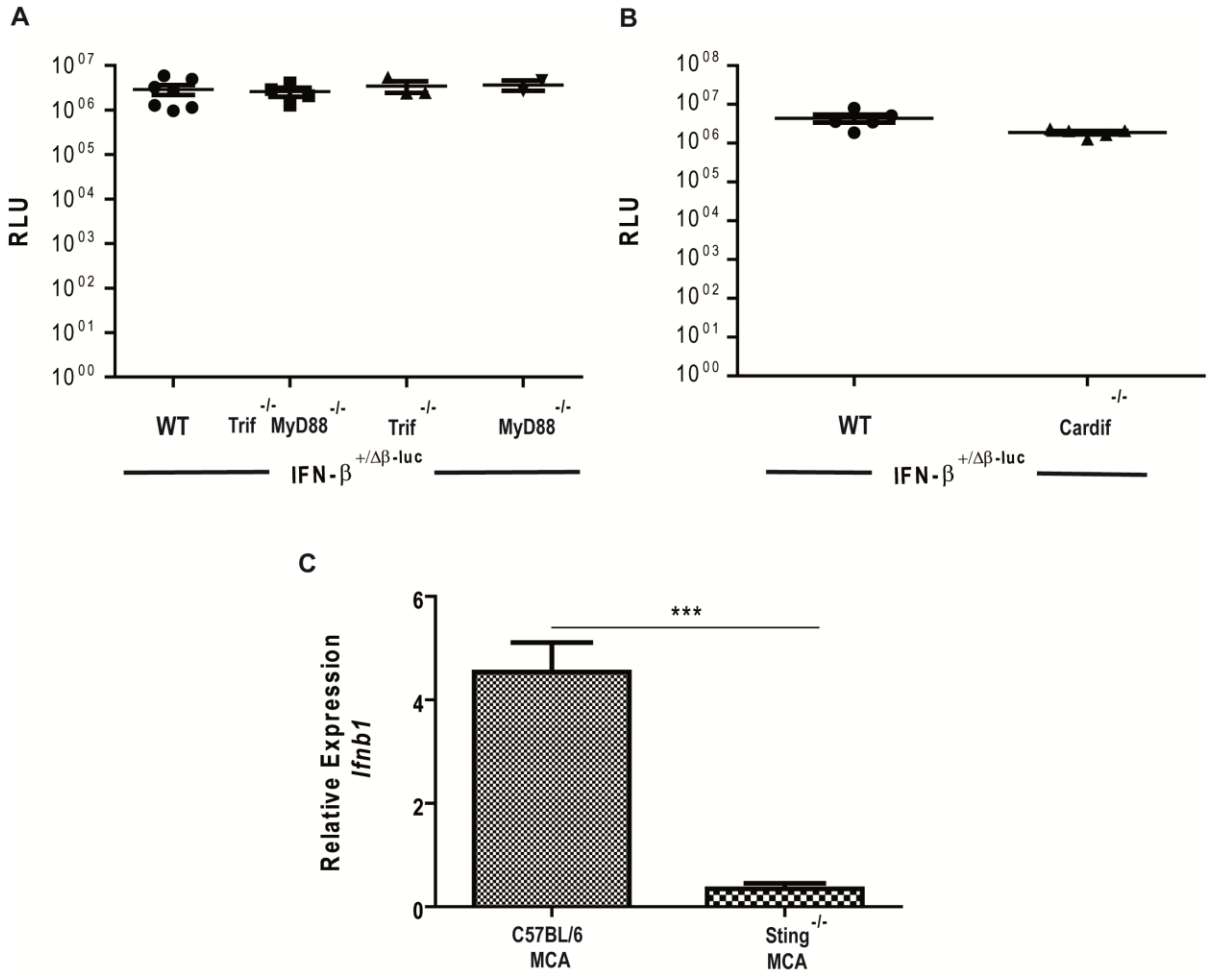

Figure 4.6. Identification of the signaling pathways inducing type I IFN expression in tumors. To identify the receptor system and transcription factors involved in IFN-β induction under tumor conditions, a combination of knockout mutants for the respective proteins and the IFN-β reporter mouse approach was used. Therefore, MCA205 fibro sarcoma were inoculated s.c. into the back of global reporter and reporter animals deficient for the TLR adaptor proteins Trif and MyD88 (A) or the RLR adaptor protein Cardif (B). On day 14 after tumor cell injection the mice were sacrificed and *ex vivo* luciferase measurement was performed for tumor homogenates. The role of CDR signaling in type I IFN induction was assessed using qRT-PCR for IFN-β gene expression in total tumor tissue of C57BL/6 wild type mice or *Sting*$^{-/-}$ animals.(C). The data represent a mean of at least 4 animals per group. The experiments were repeated at least once and statistical significance was calculated using unpaired students t test or one way ANOVA with turkey post-test (*p≤0,05).

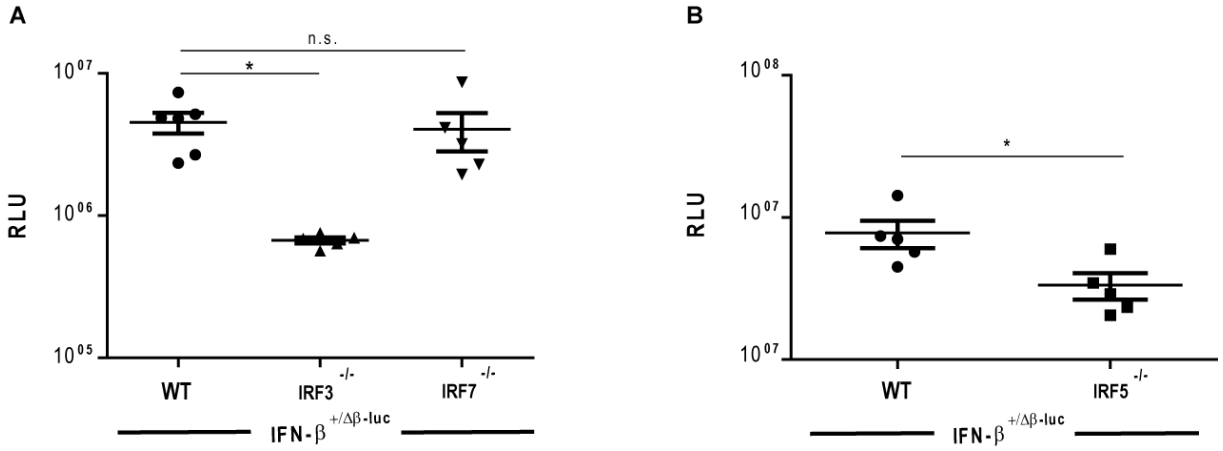

Figure 4.7. Identifikation of the signaling pathways inducing type I IFN expression in tumors. To identify the receptor system and transcription factors involved in IFN-β induction under tumor conditions a combination of knockout mutants for the respective proteins and the IFN-β reporter mouse approach was used in the present work. Therefore, MCA205 fibro sarcoma were inoculated s.c. into the back of global reporter animals and reporter animals deficient for or the IRF family members IRF3, IRF7 (A) or IRF5 (B). The data represent a mean of at least 4 animals per group. The experiments were repeated at least once and statistical significances were calculated using unpaired students t test or one way ANOVA with turkey post-test (*p≤0,05).

Regarding the IRF transcription factors, the absence of IRF7 did not alter the bio luminescent signal *ex vivo*, (Fig.4.7. A). The lack of IRF3 on the other hand reduced the signal drastically. However, the signal was not completely abolished upon IRF3 deficiency, suggesting that additional transcription factors can independently of IRF3 induce IFN-β gene expression. Indeed, IRF5 deficiency significantly decreased the reporter signal and therefore seems to be involved in IFN-β induction under tumor conditions (Fig.4.7. B).

Taken together these results demonstrate that type I IFN induction in solid tumors depends on the adaptor protein Sting and the transcription factors IRF3 and IRF5.

4.2. Influences of endogenous IFN-β on life span and cell death of TAN

As already described in previous studies, endogenous type I IFNs influence growth, angiogenesis and metastasis formation of different transplantable murine tumors via the regulation of TAN characteristics and migration (Jablonska et al., 2013, 2010). In addition type I IFNs were demonstrated to regulate maturation and apoptosis of various other cell subsets including DCs, macrophages and T cells (González-Navajas et al., 2012). Interestingly, detailed analysis of type I IFN mediated effects on neutrophil maturation, life span and apoptosis under tumor conditions are lacking to date. Therefore, the second part of the present work aimed to elucidate the impact of endogenous type I IFNs on the maturation, turn over and apoptosis of neutrophilic granulocytes in a tumor setting.

4.2.1. Endogenous IFN-β controls neutrophil turn over and maturation under tumor conditions

Under steady state conditions, neutrophil maturation, turn over and apoptosis are tightly regulated and circulation time as well as life span of neutrophilic granulocytes are comparably short (Amulic et al., 2012a). Interestingly, under inflammatory conditions these features appear to be significantly altered and neutrophil life span can be remarkably prolonged (Mayadas et al., 2014; Amulic et al., 2012a; Elbim and Estaquier, 2010). This is also true for tumor bearing hosts, where altered neutrophil kinetics can be observed and seem to be associated with adverse prognostic outcomes for the patients (Fridlender and Albelda, 2012). Of note, investigation of the complex regulation underlying neutrophil kinetics under tumor conditions is just emerging and little is known about the role of type I IFNs in this scenario. Therefore, in the present work neutrophil turn over and maturation under tumor conditions were analyzed in mice sufficient or deficient for endogenous IFN-β. To this end, tumor bearing animals were injected i.v. with BrdU to monitor neutrophil turn over via flow cytometry as depicted in figure 4.8. (A). Moreover, the maturation status of circulating blood neutrophils was assessed using phenotypic cytospin analysis of sorted blood neutrophils from the various mice (Fig.4.8. B to D).

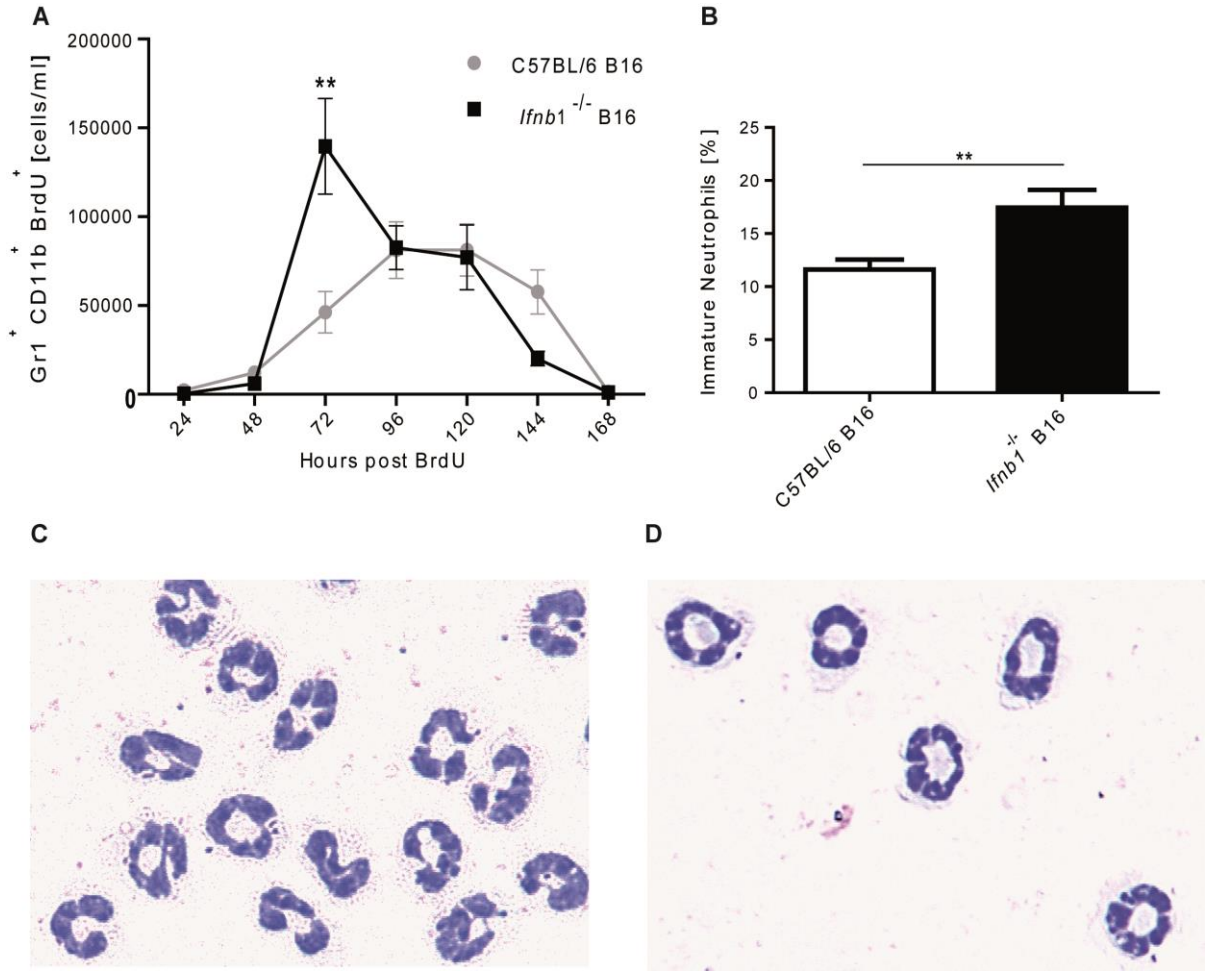

Figure 4.8. Altered mobilization and maturation of neutrophilic granulocytes under tumor conditions in the absence of endogenous IFN-β. To investigate neutrophil turn over in presence or absence of endogenous IFN-β C57BL/6 and syngeneic *Ifnb1*$^{-/-}$ mice were challenged with B16F10 melanoma. On day 7 after tumor inoculation the mice were i.v. injected with BrdU (1 mg) and the occurrence of Gr1^{+} CD11b^{+} BrdU^{+} labeled neutrophils in blood was monitored for 7 days via flow cytometry. In addition, neutrophils from blood of the different animals were sorted on day 14 after tumor inoculation and cytospin analysis were performed (B). Panels (C) and (D) illustrate the representative morphological maturation levels of neutrophils derived from blood of WT (C) and syngeneic *Ifnb1*$^{-/-}$ (D) tumor bearing mice. The experiment was repeated twice with at least 5 mice per group and statistical significance was determined using unpaired students t test or two way ANOVA (**p≤0,005).

Interestingly, in tumor bearing *Ifnb1*$^{-/-}$ mice, turn over and mobilization of Gr1^{+} CD11b^{+} BrdU^{+} labeled neutrophils was significantly faster. Furthermore, a higher number of neutrophils entered the blood stream under such conditions (Fig.4.8. A). This was most likely due to the release of immature neutrophils into circulation in tumor bearing mice deficient for

endogenous IFN-β. Significantly elevated numbers of neutrophils with a ring-shaped nucleus, indicative for immature neutrophils, could be detected via cytospin analysis (Fig.4.8. B to D).

4.2.2. Prolonged life span of neutrophilic granulocytes from IFN-β deficient mice

Neutrophil counts, turn over and maturation in *Ifnb1*$^{-/-}$ mice are significantly altered, compared to wild type control mice (Jablonska et al., 2010). At steady state, the life span of neutrophils is short, but increases under pro-inflammatory conditions. Extrapolating these data, the life span of TANs might be increased in the absence of IFN-β. This could be one reason for the accumulation of such cells in tumors. To proof this hypothesis, C57BL/6 or BALB/c and syngeneic *Ifnb1*$^{-/-}$ mice were challenged with B16F10 melanoma or 4T1 mamma carcinoma cells, respectively. To assess their live span, neutrophils from blood and tumors were isolated to high purity on day 14 after tumor inoculation and cultivated for 18 hours *ex vivo*. Subsequently, the cells were stained with propidium iodide (PI) to distinguish live and dead cells (Fig.4.9.).

As shown in Figure 4.9., neutrophils derived from blood and tumors of IFN-β deficient mice exhibited a significantly prolonged life span, compared to wild type controls in both tumor models (Fig.4.9. A to D). This phenomenon was partly abolished when small amounts of rmIFN-β were added to the cultures (Fig.4.9. A and B).

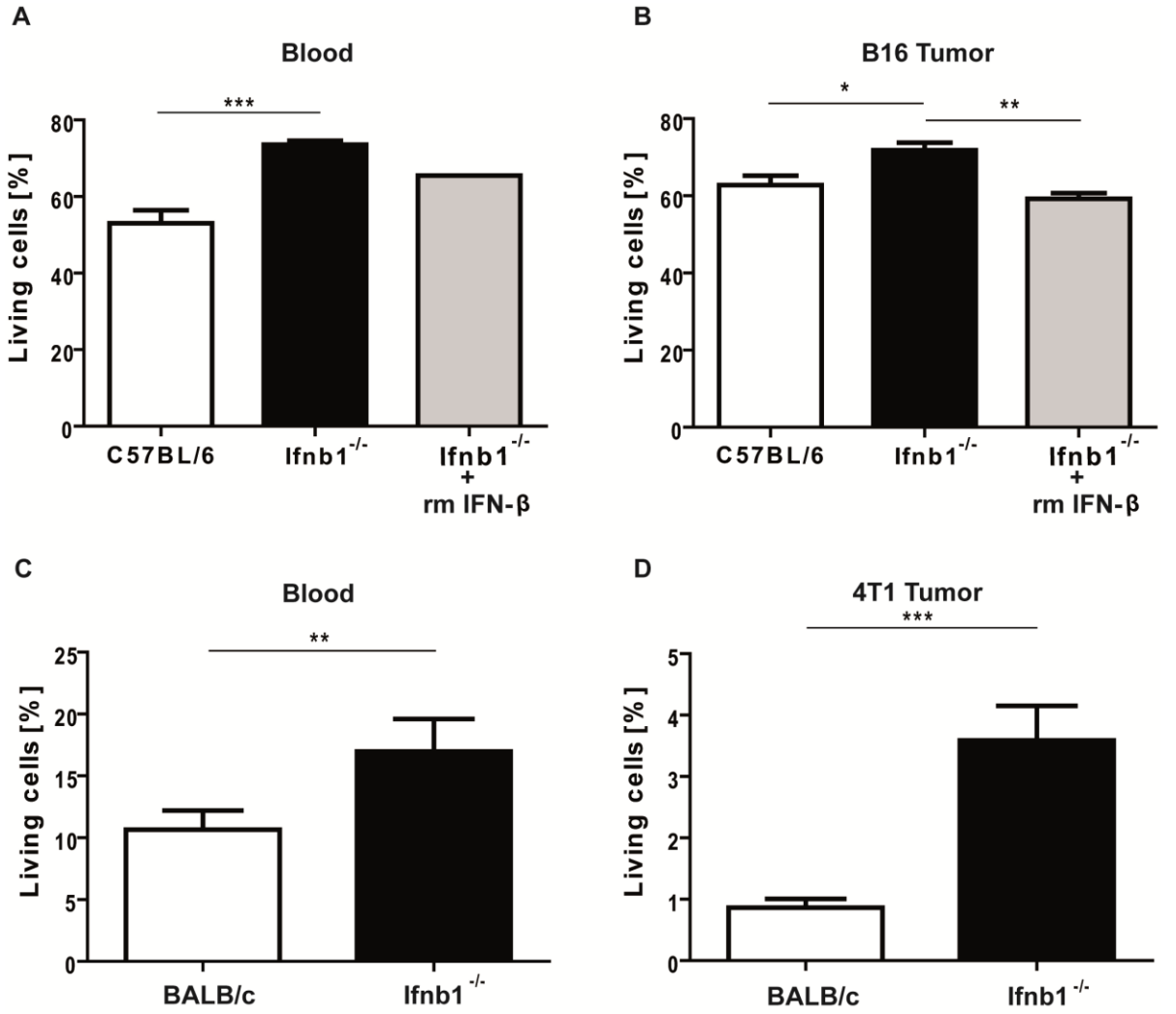

Figure 4.9. Neutrophil life span is prolonged in the absence of endogenous type I IFN. Percentage of living CD11b^{hi} Gr1^{hi} neutrophil granulocytes derived from (A) blood and (B) tumor of *Ifnb1*$^{-/-}$ C57BL/6 mice or (C) blood and (D) tumor of *Ifnb1*$^{-/-}$ BALB/c mice is increased, after 18h in culture *ex vivo,* in comparison to controls. 4T1 breast carcinoma cells were injected s.c. into the mammary fat pad of BALB/c or *Ifnb1*$^{-/-}$ mice. C57BL/6 mice were injected s.c. on the flank with B16F10 melanoma cells. After 14 days mice were sacrificed, total cell suspensions of blood and tumor prepared and neutrophils isolated using FACS Aria sorter. Subsequently, cells were cultivated *ex vivo* for 18 hours in RPMI, stained with PI and analyzed using the BD LSR II system. Data represent mean ± SEM. (* $p \leq 0.01$).

4.2.3. Decreased Fas expression on neutrophils from tumor bearing *Ifnb1*$^{-/-}$ mice

Apoptosis is the predominant pathway of neutrophil cell death and can be induced via death receptor signaling (Jonsson et al., 2005). In line with the prolonged life span of neutrophils in the absence of endogenous IFN-β, decreased expression of Fas on neutrophils derived from

tumors (Fig.4.11. B) and lung (Fig.4.11. C) was observed. Blood derived neutrophils showed equivalent Fas expression in wild type and *Ifnb1*$^{-/-}$ mice, suggesting that Fas regulation by IFN-β takes place after the second transmigration into target tissues (Fig.4.11. A). Similar results were obtained for wild type and *Ifnb1*$^{-/-}$ mice on BALB/c background (Fig.4.10). Of note, since the *ex vivo* life span of blood neutrophils from tumor bearing *Ifnb1*$^{-/-}$ mice is prolonged without a significant upregulation of death receptors (Fig.4.11. A; Fig.4.10 A), additional regulatory mechanisms independent of death receptor signaling might be involved.

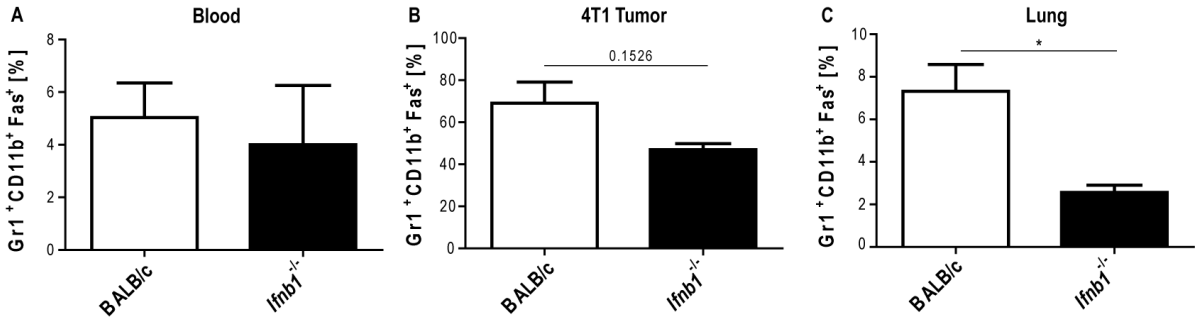

Figure 4.10. Neutrophil granulocytes from *Ifnb1*$^{-/-}$ tumor bearing mice show decreased expression of Fas independent of the tumor model. 4T1 tumors were inoculated s.c. into the mammary fat pad of either BALB/c WT or *Ifnb1*$^{-/-}$ mice. 14 days after tumor injection the mice were sacrificed and single cell suspensions of (A) blood, (B) tumor and (C) lung were prepared and analyzed using the BD LSR II system. Depicted is the quantification of Fas expression on CD11b^{hi} Gr1^{hi} Ly6C ^{int} granulocytes. Three independent experiments were performed, with at least 5 mice per group. Statistical significance was determined using unpaired Students t-test (** $p \leq 0{,}005$).

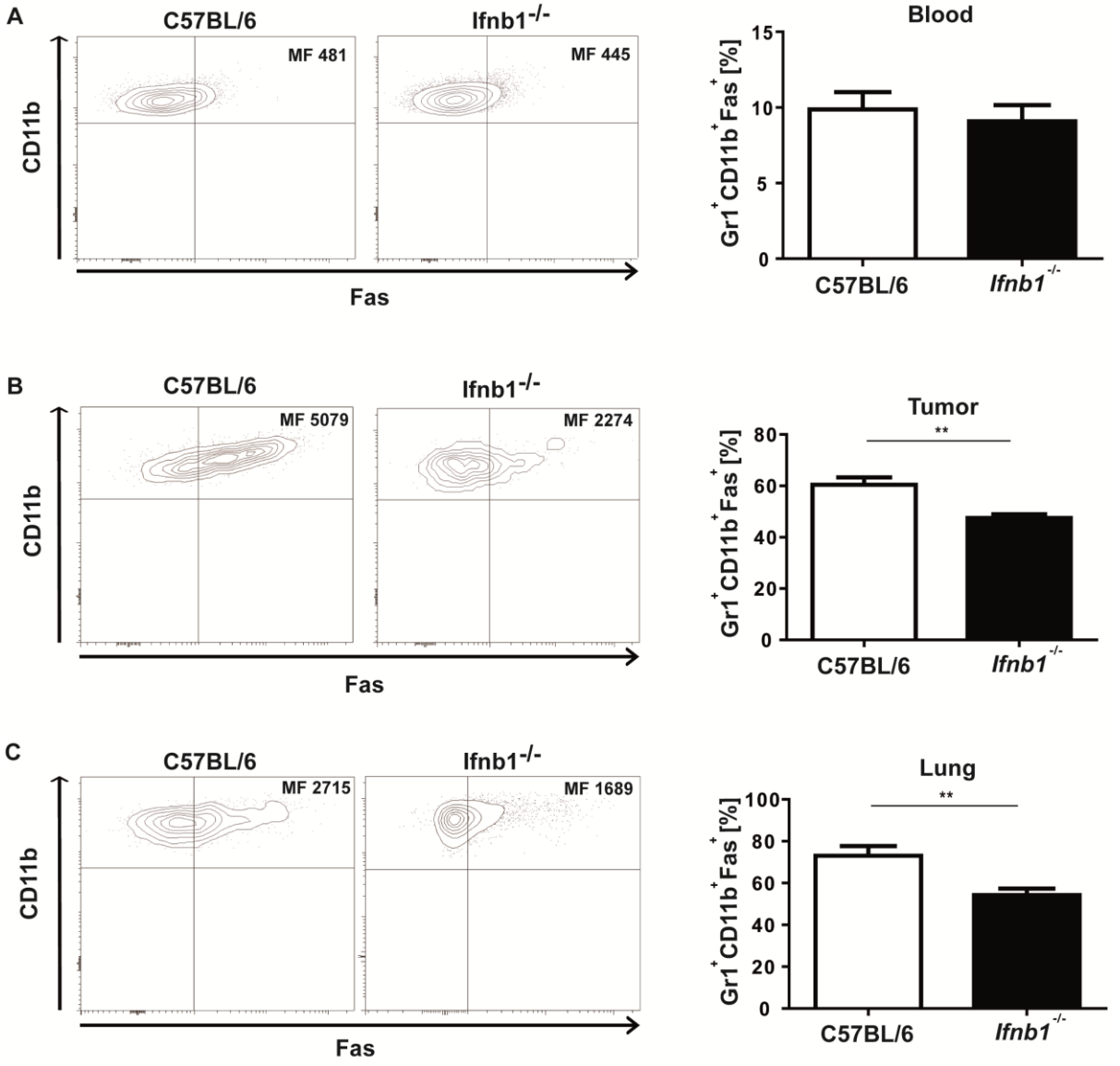

Figure 4.11. Neutrophil granulocytes from *Ifnb1*$^{-/-}$ tumor bearing mice show decreased expression of Fas after their transmigration into the target tissues. B16F10 tumors were inoculated s.c. on the back of either C57BL/6 WT or *Ifnb1*$^{-/-}$ mice. 14 days after tumor injection the mice were sacrificed and single cell suspensions of (A) blood, (B) tumor and (C) lung were prepared and analyzed using the BD LSR II system. Depicted are representative FACS plots and quantification of Fas expression on CD11b^{hi} Gr1^{hi} Ly6C ^{int} granulocytes. Three independent experiments were performed, with at least 5 mice per group. Statistical significance was determined using unpaired Students t-test (** $p \leq 0{,}005$).

4.2.4. Diminished ROS production by neutrophils in the absence of endogenous IFN-β

Besides apoptosis induction via cell-cell contact, neutrophils harbor intrinsic activities involved in the regulation of this process. Upon stimulation, they produce huge amounts of ROS that kill invading microbes (Amulic et al., 2012b). A large body of evidence suggests that ROS can also promote neutrophil apoptosis via a not yet defined mechanism (Gabelloni et al., 2013). To address whether ROS production is reduced in the absence of IFN-β in a tumor bearing host and thus influences the neutrophil life span, C57BL/6 and syngeneic *Ifnb1*$^{-/-}$ mice were challenged with B16F10 melanoma cells. Animals were sacrificed 14 days after tumor inoculation and single cell suspensions from blood and tumors were tested for their content of ROS, using the cell-permeable reagent 2´, 7´-dichlorfluorescein diacetate (DCFDA). Interestingly, neutrophils isolated from IFN-β deficient mice showed reduced ROS production, compared to wild type controls (Fig. 4.12. A and C). This difference was especially significant in tumor infiltrating neutrophils (Fig.4.12. C).

To further confirm the importance of ROS for neutrophil apoptosis, neutrophils from the different tissues of C57BL/6 or syngeneic *Ifnb1*$^{-/-}$ mice were isolated, as described above, and incubated for 18 hours in the presence or absence of apocyanin or taurine to inhibit generation of ROS. Afterwards, the cells were stained with PI and Annexin V to detect apoptotic cells by flow cytometry. Apparently, the NADPH oxidase inhibitor apocyanin significantly reduced the apoptosis rate of neutrophils derived from blood (Fig.4.12. B) and tumor (Fig.4.12. D) of both types of mice. The same holds true for the ROS scavenger taurin in blood derived neutrophils of C57BL/6 mice (Fig.4.12. B).

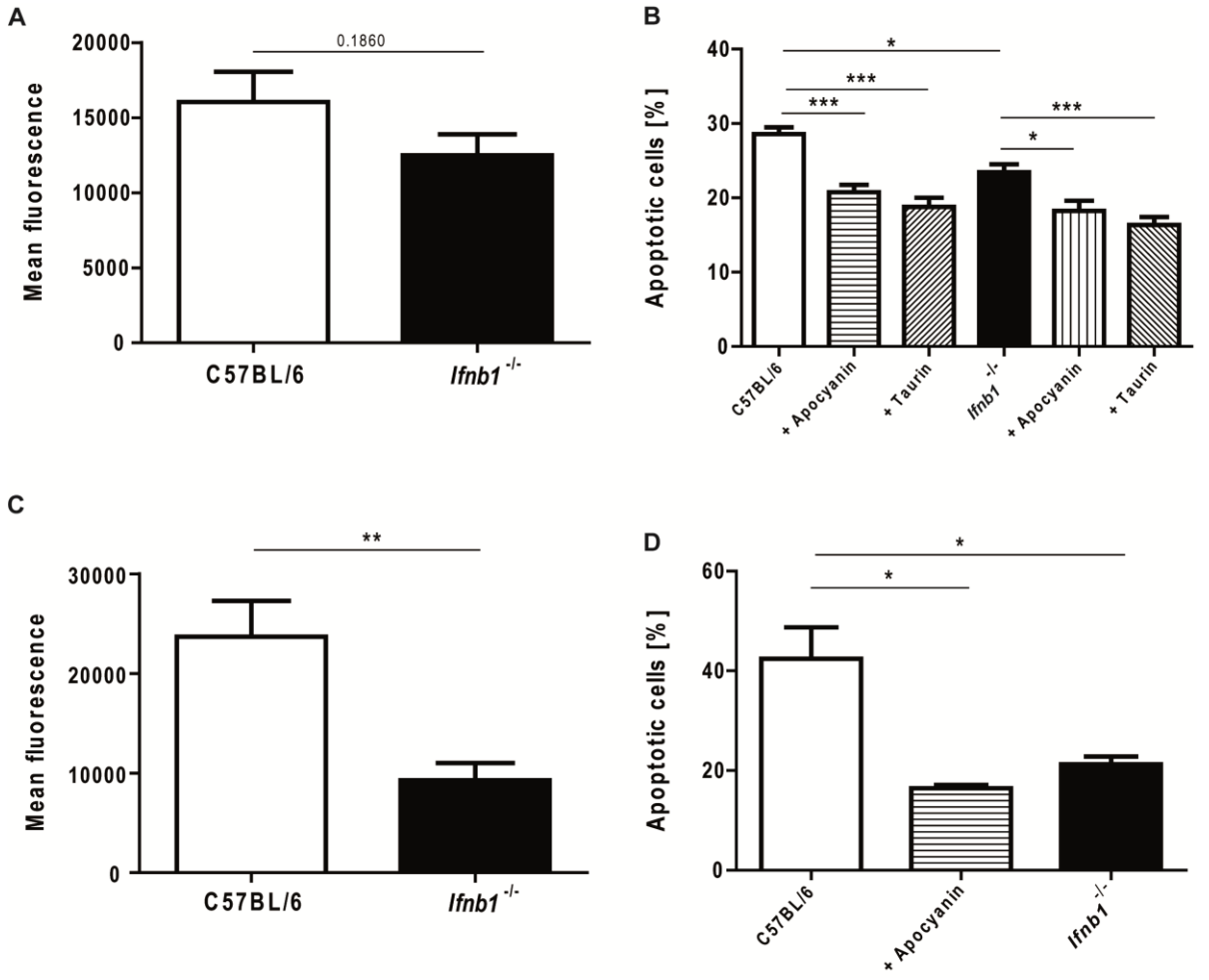

Figure 4.12. Tumor-infiltrating neutrophils produce lower amounts of cytotoxic ROS in the absence of endogenous IFN-β. B16F10 melanoma cells were s. c. injected on the back of C57BL/6 mice or *Ifnb1*$^{-/-}$ mice. 14 days after tumor inoculation the mice were sacrificed, single cell suspensions of tumors and lungs prepared, and stained for CD11b and Ly6G. Afterwards, cells were incubated in RPMI medium containing 20 µM 2´, 7´-dichlorfluorescein diacetate (DCFDA). Depicted is the ROS production of blood (A) and tumor (C) derived CD11b^{hi} Ly6G ^{hi} neutrophil granulocytes. To additionally verify the role of ROS in neutrophil apoptosis, CD11b^{hi} Ly6G ^{hi} neutrophil granulocytes from blood (B) and tumor (D) were isolated using BD FACS Aria for cell sorting. Cells were incubated 18 hours in RPMI with or without the addition of apocyanin (100 µM) or taurin (50 mM). Thereafter, the cells were stained with PI and Annexin V, and analyzed using the BD LSR II system. Data represent mean ± SEM. (* $p \leq 0.01$).

4.2.5. IFN-β regulates the expression of pro- and anti-apoptotic BCL2 family members in tumor- and blood-derived neutrophils

The intracellular apoptosis pathway is tightly regulated by several members of the Bcl-2 family. This proteins are characterized by their pro- or anti-apoptotic functions as well as by their expression of the Bcl-2 homology domain (García-Sáez, 2012). For neutrophils, the expression of the pro-apoptotic Bcl-2 protein Bax and its anti-apoptotic counterpart BCL-xL is of special importance (Weinmann et al., 1999; Perskvist et al., 2002).

To address whether IFN-β regulates the expression of such proteins and thus influences longevity of neutrophils, $CD11b^+$ $Gr1^+$ cells from blood and tumors of B16F10 tumor-bearing mice were sorted and mRNA isolated to perform quantitative Real-Time-PCR (qRT-PCR). In accordance with increased longevity, tumor infiltrating neutrophils from *Ifnb1*$^{-/-}$ mice exhibited a significantly increased BCL-xL expression accompanied by only slightly increased Bax expression. In contrast, tumor infiltrating neutrophils from wild type mice show the opposite pattern of expression (Fig.4.13. A and B). Pre-incubation of *Ifnb1*$^{-/-}$ tumor-infiltrating neutrophils with 5 units of rmIFN-β partly restored WT gene expression. Additionally, in tumor neutrophils of WT animals the Bax/BCL-xL ratio was highly up-regulated, compared to *Ifnb1*$^{-/-}$ mice (Fig.4.13. C). Interestingly, in blood neutrophils expression levels as well as Bax/BCL-xL ratio were reduced compared to neutrophils from tumors. This is consistent with the idea that mainly tissue resident neutrophils undergo apoptosis.

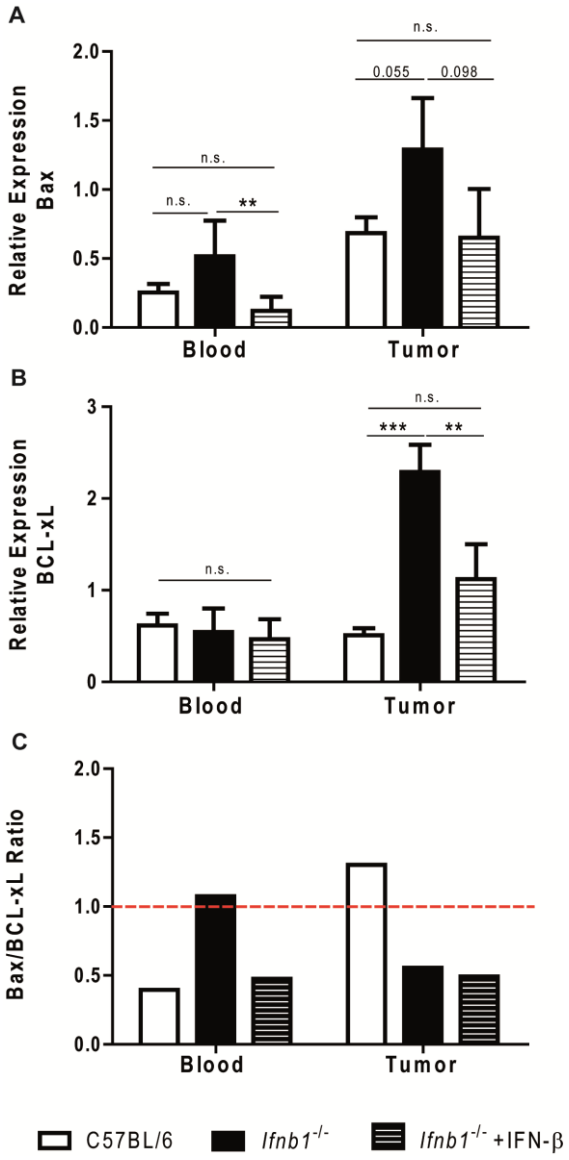

Figure 4.13. Bax and BCL-xL gene expression in blood and tumor neutrophils is regulated by IFN-β. Tumors and blood were harvested at day 14 after tumor inoculation, single cell solutions prepared, stained and $CD11b^{+}Gr1^{+}$ neutrophils were sorted out. RNA was isolated, cDNA prepared and gene expression of Bax (A) and BCL-xL (B) measured using quantitative RT-PCR. To visualize a pro- or anti-apoptotic expression pattern of these two Bcl-2 family members the Bax/BCL-xL ratio was assessed (C). Cells were derived from five pooled animals. All experiments were repeated at least one time.

4.2.6. Altered expression of major apoptosis regulators and apoptosome components in the absence of endogenous IFN-β

As a consequence of Bax dependent mitochondrial outer membrane permeabilization, cytochrome c is released and apoptosome formation is induced. Apaf1 oligomerizes and the

apoptosome accomplishes cleavage and activation of pro-caspase 9, an initiator caspase that induces processing of downstream caspases (Li et al., 1997; Slee, 1999). To investigate the role of apoptosome components in IFN-β dependent apoptosis of neutrophils derived from blood (Fig.4.14. A and B) or tumor (Fig.4.14. C and D), C57BL/6 and *Ifnb1*$^{-/-}$ mice were challenged s.c. with B16F10 melanoma cells. On day 14 after tumor inoculation, neutrophils were isolated from the different tissues and qRT-PCR analysis were performed.

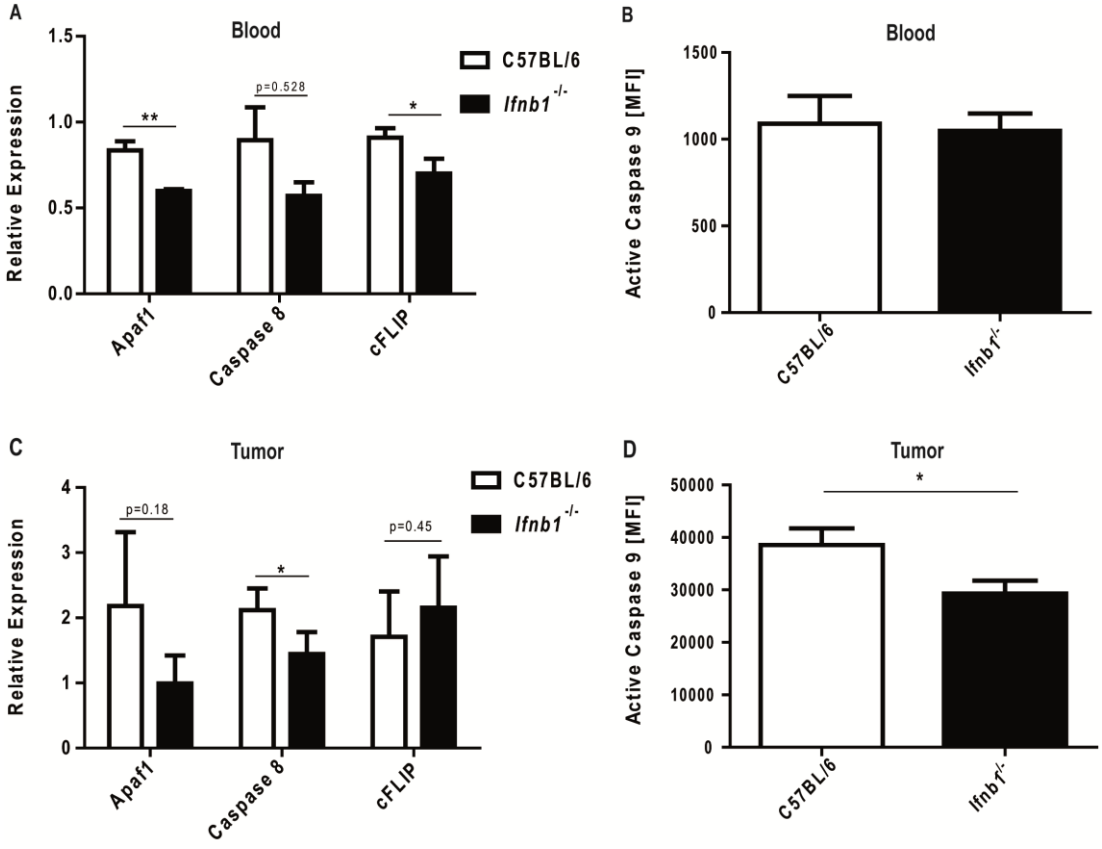

Figure 4.14. Altered expression of major regulators of apoptosis and apoptosome constituents in the absence of endogenous IFN-β. To evaluate expression level of Apaf1, caspase 8 and cFLIP, mice were s.c. injected with B16F10 tumor cells. On day 14 after tumor inoculation Ly6G^{+} CD11b^{+} neutrophils from (A) blood and (C) tumor of wild type or syngeneic *Ifnb1*$^{-/-}$ mice were sorted using a BD FACS Aria system, mRNA was isolated and quantitative RT-PCR analyses performed. Data represent pools of at least 6 mice per group. In addition, the caspase 9 activity was assessed in Ly6G^{+} CD11b^{+} neutrophils from (B) blood and (D) tumor by FACS analysis, using FITC coupled caspase inhibitor FAM-FLICA. The experiment was repeated twice with at least 5 mice per group and statistical significance was determined using unpaired students t test or one way ANOVA with turkey posttest (*p≤0,05).

Apparently, Apaf-1 and caspase 8 expression was downregulated in the absence of endogenous IFN-β in neutrophils from blood (Fig.4.14 A) and tumor (Fig.4.14 B). Besides, also the FADD-like interleukin-1β-converting enzyme (FLICE)-inhibitory protein (cFLIP), a major anti-apoptotic regulator (Safa, 2012), could be involved in the altered apoptosis rate of *Ifnb1*$^{-/-}$ neutrophils. Interestingly, cFLIP expression was significantly higher in blood derived neutrophils from wild type mice (Fig.4.14 A), but no differences were observed in TAN (Fig.4.14 B).

Additional analysis of neutrophils for cytochrome c release (Fig.4.14) and caspase 9 activity (Fig.4.14 B and D) revealed no significant changes regarding blood neutrophils from IFN-β sufficient or deficient tumor-bearing mice (Fig.4.14 B; Fig.4.14 A and B). In contrast, tumor derived neutrophils of *Ifnb1*$^{-/-}$ mice displayed reduced caspase 9 activity (Fig.4.14 D) compared to wild type controls, although no differences in cytochrome c release could be detected (Fig.4.15 C and D).

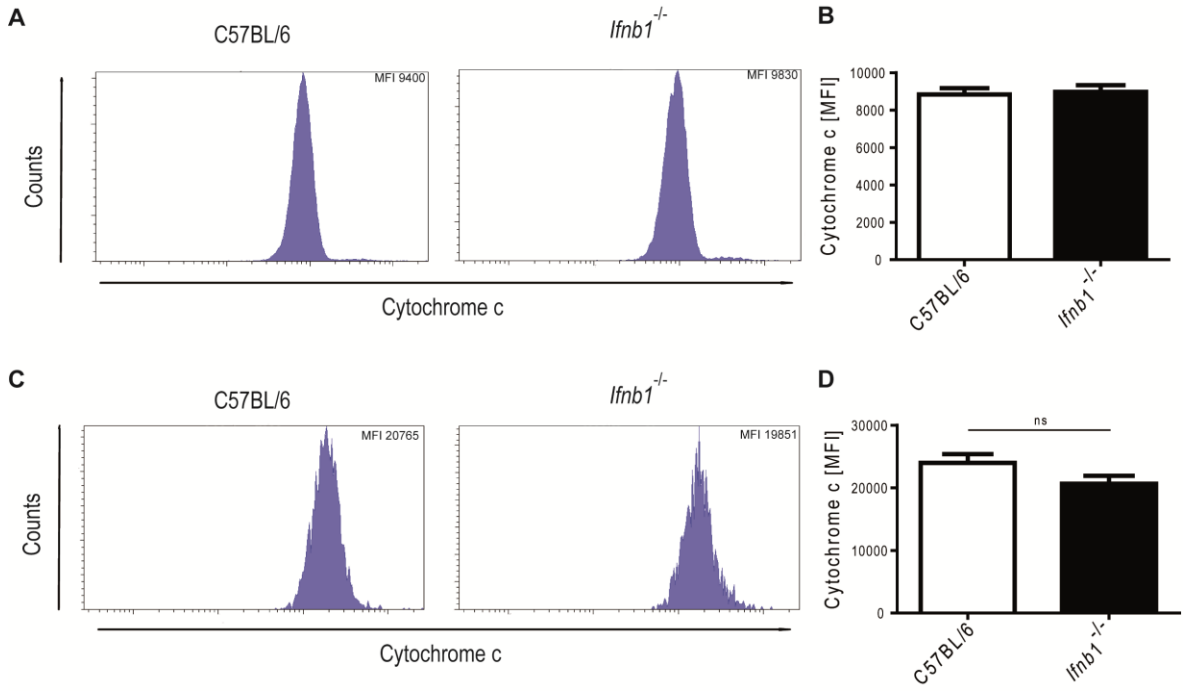

Figure 4.15. Cytochrome c release of neutrophils is not influenced by endogenous IFN-β. To assess the cytochrome c release of mitochondria in neutrophil granulocytes, B16F10 melanoma cells were injected s.c. on the back of C57BL/6 and syngeneic *Ifnb1*$^{-/-}$ mice. On day 14 after tumor inoculation single cell suspensions of blood (A and B) and tumor (C and D) were prepared and stained for Ly6G, CD11b and cytochrome c. No significant differences were detected.

4.2.7. Reduced pro-apoptotic signaling in neutrophils of tumor bearing *Ifnb1*$^{-/-}$ mice decreases effector caspase activity

Caspases are crucial for the initiation, propagation, and execution of apoptosis. Neutrophils express a set of six pro-apoptotic caspases, three initiator caspases (8, 9, and 10) and three effector caspases (3, 6 and 7) (Chang and Yang, 2000). They are activated via two main pathways: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway induced by DNA damage or oxidative stress (Galluzzi et al., 2012b; Degterev and Yuan, 2008). Upon activation, pro-caspases are cleaved leading to the formation of mature caspases (Chang and Yang, 2000). Since important pro-apoptotic stimuli, as well as initiator caspase expression (Fig.4.14 A and C) and activity (Fig.4.14 B and D) were altered in neutrophils of tumor bearing *Ifnb1*$^{-/-}$ mice, it was analyzed whether the expression or activation level of the effector caspase 3 is also affected.

Therefore, neutrophils from blood, tumor and lung of mice bearing B16F10 melanoma were tested for the expression of caspase 3 using qRT-PCR. A strong reduction of caspase 3 expression was detected in neutrophils of tumor bearing *Ifnb1*$^{-/-}$ mice, compared to control animals. This was true for all three anatomical compartments (Fig.4.16 A), although expression levels were dramatically lower in neutrophils from blood and lung, compared to tumors.

To address the post-transcriptional regulation of caspase 3, the status of the active form of this protease was assessed. As expected, tumors of wild type animals displayed the highest percentage of neutrophils containing active caspase 3 (Fig.4.16 B and C), that was significantly reduced in the absence of endogenous IFN-β. Regarding blood and lung of tumor bearing animals a lower percentage of neutrophils appeared positive for active caspase 3, compared to tumor tissue (Fig.4.16 B and C). Nevertheless, the absence of endogenous IFN-β further reduced it. Thus, consistent with the enhanced longevity of neutrophils in tumor bearing *Ifnb1*$^{-/-}$ mice, expression of mature effector caspase 3 was reduced in neutrophils from various anatomical compartments on C57BL/6 as well as BALB/c background (Fig.4.16, Fig.4.17).

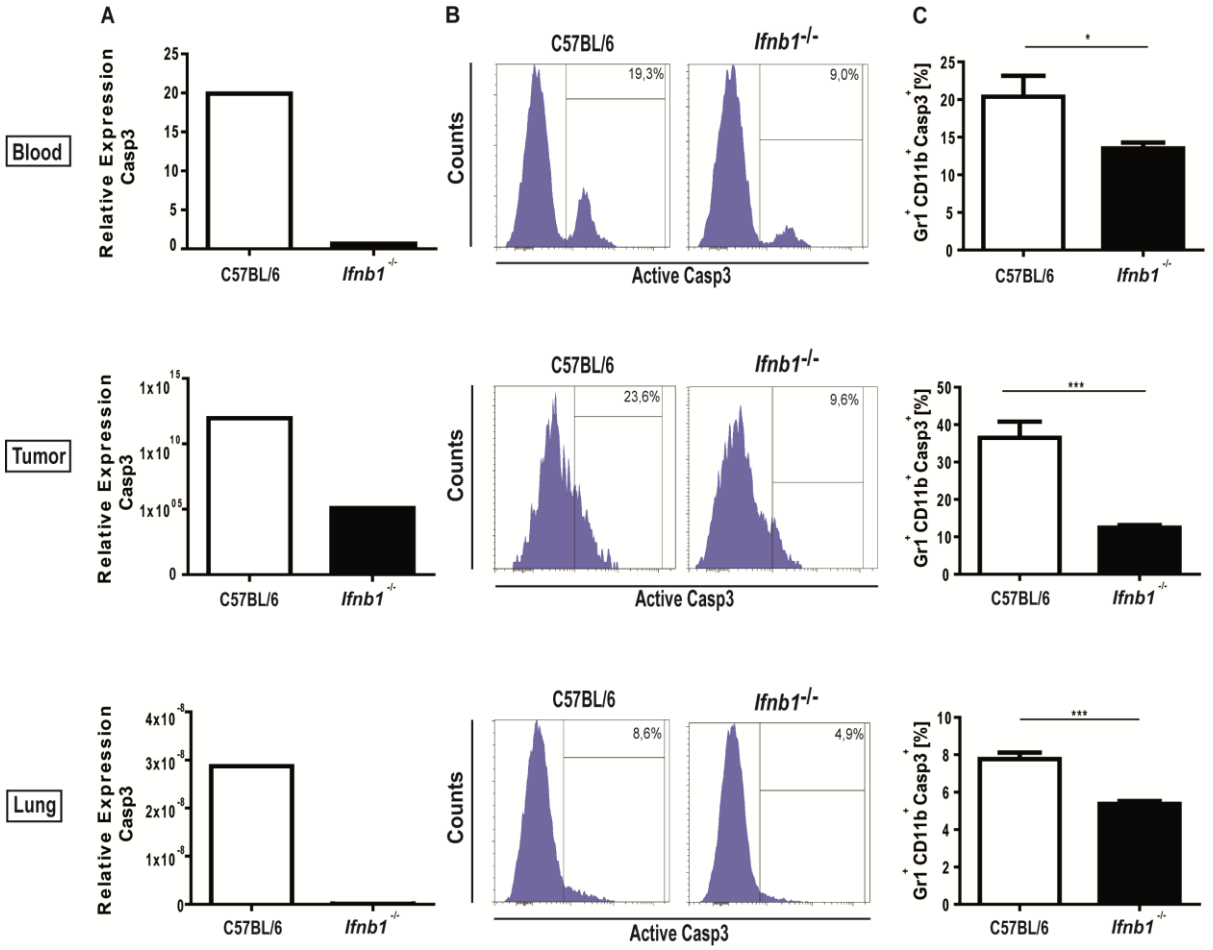

Figure 4.16. Neutrophils of *Ifnb1*$^{-/-}$ tumor bearing mice show a decreased caspase 3 activity. To assess the relative gene expression of caspase 3 (A), quantitative RT-PCR for blood, tumor and lung was performed. To measure the activity of the effector caspase 3 (B and C) in blood, tumor and lung neutrophils, B16F10 melanoma cells were s.c. injected to the back of C57BL/6 or *Ifnb1*$^{-/-}$ mice. On day 14 after tumor inoculation single cell suspensions of the different organs were prepared and analyzed using the LSR II system. The experiment was repeated two times and statistic significance was evaluated using unpaired students t test (* p≤ 0,05).

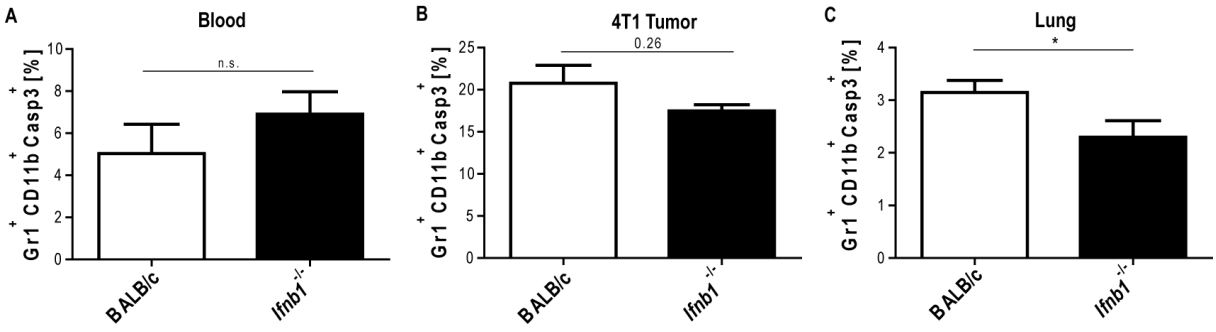

Figure 4.17. Neutrophils of *Ifnb1*$^{-/-}$ tumor bearing mice show a decreased caspase 3 activity independent of mouse strain or tumor model. To measure the activity of the effector caspase 3 in blood (A), tumor (B) and lung (C) neutrophils, 4T1 cells were s.c. injected to the mammary fat pad of BALB/c or *Ifnb1*$^{-/-}$ mice. On day 14 after tumor inoculation single cell suspensions of the different organs were prepared and analyzed using the LSR II system. The experiment was repeated two times and statistic significance was evaluated using unpaired students t test (* p≤ 0,05).

4.2.8. G-CSF expression is increased in the absence of endogenous IFN-β

Neutrophil life span and survival are also regulated by growth factors like Granulocyte Colony Stimulating Factor (G-CSF). To further investigate the mechanisms underlying the prolonged life span of neutrophils in tumor bearing *Ifnb1*$^{-/-}$ mice, the serum levels of G-CSF was analyzed by ELISA in presence and absence of endogenous IFN-β. Additionally, G-CSF expression in neutrophils was assessed by qRT-PCR.

Interestingly, the relative expression of G-CSF in neutrophils derived from blood, tumor and lung of tumor bearing *Ifnb1*$^{-/-}$ mice was remarkably increased, in comparison to wild type animals, but apparently not influenced in spleen (Fig. 4.18). Expression levels were strongly reduced when *Ifnb1*$^{-/-}$ neutrophils were incubated with low amounts of rmIFN-β (Fig.4.18 A). Congruent results were obtained for serum G-CSF. On day 14 after tumor injection, *Ifnb1*$^{-/-}$ mice showed a significantly increased concentration of G-CSF in serum (Fig. 4.18 E).

The G-CSF receptor signals via the PI3K/Akt pathway to inhibit the pro-apoptotic Bcl-2 family member Bax. To test whether the enhanced serum G-CSF level observed in the absence of endogenous IFN-β has an impact on G-CSF signaling in tumor tissue, the phosphorylation status of PI3 kinase was analyzed. A drastic increase of pPI3K was detected in IFN-β deficient mice, compared to controls (Fig.4.18 F). Taken together, this indicates an

impact of IFN-β on the regulation of G-CSF expression underlining the important role of type I IFN in the context of neutrophil life cycle.

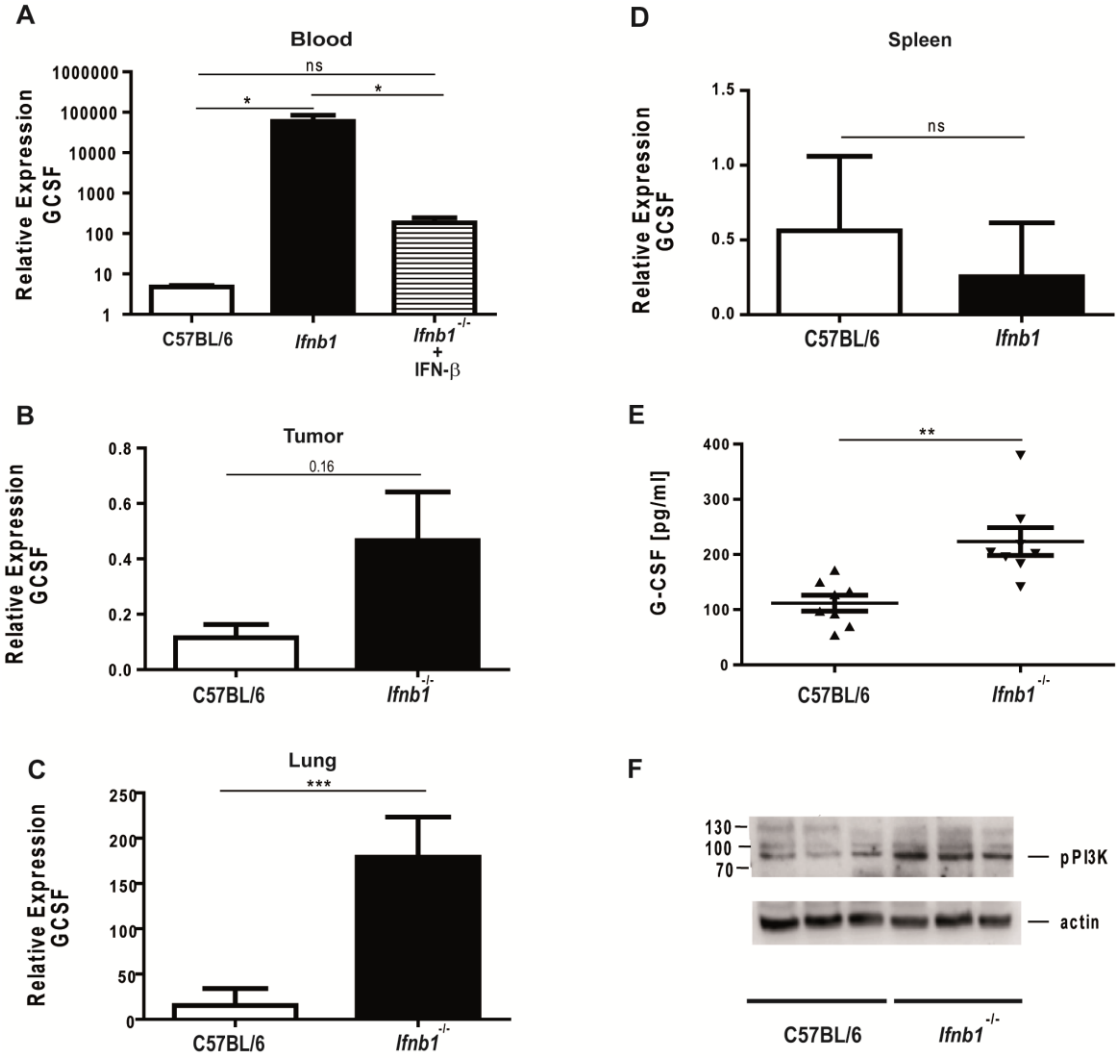

Figure 4.18. Increased G-CSF expression in the absence of endogenous IFN-β. To evaluate serum G-CSF level, mice were s.c. injected with B16F10 tumor cells. On day 14 after tumor inoculation heart blood was harvested to perform serum ELISA (E). In addition Ly6G$^+$ CD11b$^+$ neutrophils from (A) blood, (B) tumor, (C) lung and (D) spleen of tumor bearing wild type or syngeneic *Ifnb1*$^{-/-}$ mice were sorted using a BD FACS Aria system, mRNA was isolated and quantitative RT-PCR analyses performed. The important regulatory function of IFN-β was verified by 4 hours incubation of blood neutrophils in the presence or absence of recombinant IFN-β and subsequent qRT-PCR. 2 independent experiments were performed, with at least 5 mice per group. Statistical significance was determined using unpaired Students t-test (** $p \leq 0{,}005$). In addition the phosphorylation status of the G-CSF signal transducer PI3 kinase (F) was analyzed by western blot for tumor tissue of C57BL/6 mice and syngeneic *Ifnb1*$^{-/-}$ mice.

4.3. Impact of endogenous IFN-β on the phenotypic polarization of TAN

The concept of dichotomous activation and polarization is well established for tumor associated macrophages that were shown to exhibit both anti-tumor (M1) as well as pro-tumor (M2) functions, depending on the respective micromilieu (Sica and Mantovani, 2012). In 2009 a comparable concept was suggested for TAN by Fridlender and colleagues who identified TGF-β as one of the factors responsible for the induction of an N2 pro-tumor neutrophil phenotype (Fridlender et al., 2009). Indeed, these pro-tumorigenic neutrophils seem to be distinct from granulocytic MDSCs or naive neutrophils (Fridlender et al., 2012) and accumulate during tumor progression (Mishalian et al., 2013). Increasing evidence suggests that IFN-β could play a role in N1 anti-tumor polarization of TAN. It massively influences tumor growth as well as angiogenesis in a neutrophil dependent manner (Jablonska et al., 2010). In addition, it regulates neutrophil chemotaxis (Jablonska et al., 2013) and influences neutrophil maturation, turn over and apoptosis. Therefore, the present work was directed to further investigate the impact of type I IFNs on TAN polarization. To this end, different characteristics of neutrophils known to be associated with either N1 anti-tumor or N2 pro-tumor function were assessed in presence or absence of endogenous IFN-β.

4.3.1. Altered expression of co-stimulatory molecules and activation markers on *Ifnb1*$^{-/-}$ TAN

One functional ability of neutrophils is the induction of an adaptive immune response, including the activation of DCs and effector T cells (Fridlender and Albelda, 2012). To this end, neutrophils are able to express different co-stimulatory molecules such as CD80, CD86 or ICAM1 (Mantovani et al., 2011). Interestingly, especially the latter one seems to be down regulated on N2 pro-tumor neutrophils (Piccard et al., 2012). To further elucidate the influence of endogenous IFN-β on the phenotype of TANs, flow cytometric analysis of ICAM1 expression were performed on day 14 after s.c. inoculation of B16F10 melanoma or 4T1 mammary carcinoma in wild type or *Ifnb1*$^{-/-}$ animals (Fig.4.19).

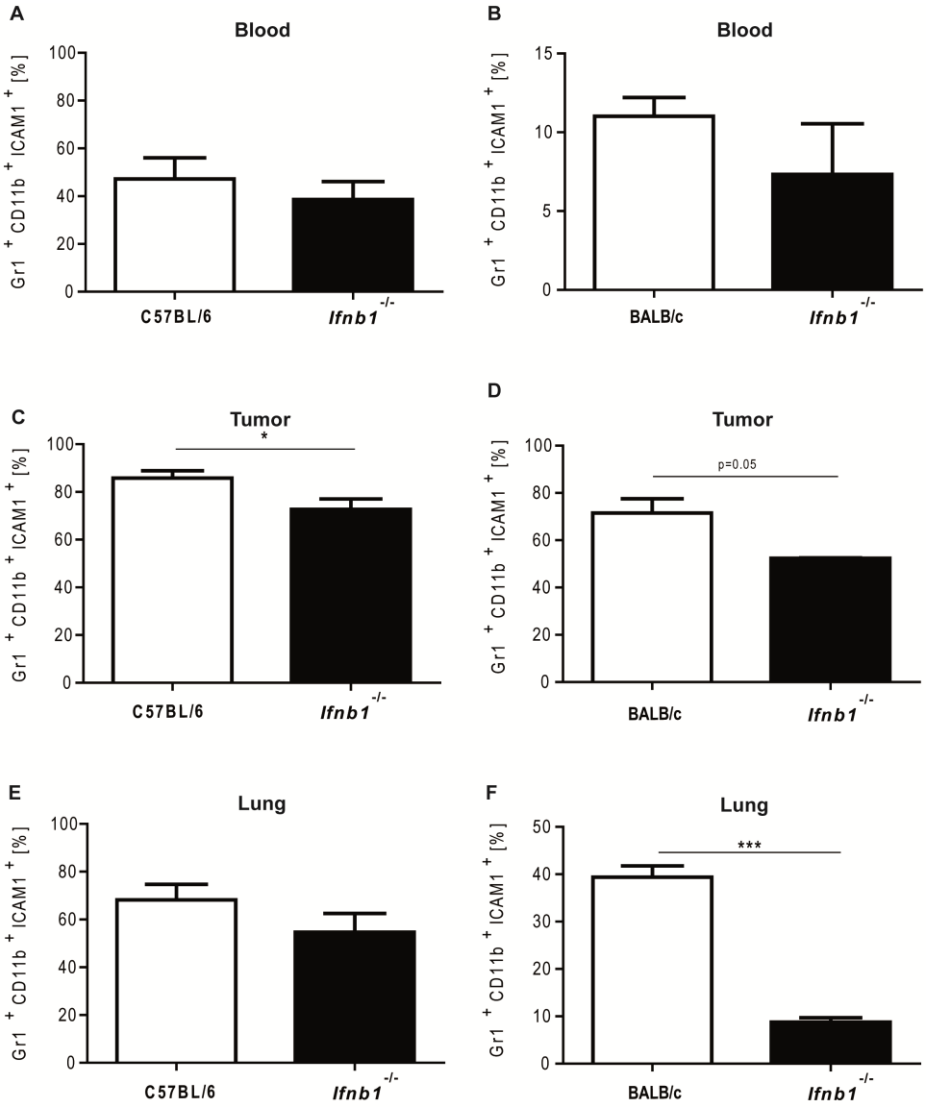

Figure 4.19. ICAM1 expression is downregulated on tissue neutrophils in the absence of endogenous IFN-β. To assess the expression of the co-stimulatory adhesion molecule ICAM1 on neutrophils from different sources, C57BL/6 and BALB/c mice, as well as syngeneic *Ifnb1*$^{-/-}$ animals, were challenged with B16F10 or 4T1 tumors, respectively. On day 14 after tumor inoculation, single cell suspension of blood (A, B), tumor (C, D) and lung (E, F) were prepared and analyzed for ICAM1 expression on CD11b^{+} GR1^{+} neutrophilic granulocytes via flow cytometry. The experiment was repeated twice with at least five mice per group and statistical significance was calculated using unpaired students t test (*p≤0,05).

No significant changes were observed for blood neutrophils in presence or absence of endogenous IFN-β (Fig.4.19. A and B). Of note, the expression of ICAM1 was remarkably upregulated after neutrophil transmigration into the target tissue (Fig.4.19. C to F). This upregulation was significantly reduced in the absence of endogenous IFN-β in tumor (Fig.4.19. C and D) as well as in lung (Fig.4.19. E and F) independent of the mouse strain.

This further validates an influence of type I IFNs on the activation and polarization of neutrophilic granulocytes.

4.3.2. Altered neutrophil activation in tumor bearing *Ifnb1*$^{-/-}$ mice

Besides ICAM1 upregulation (Fortunati et al., 2009), also the shedding of L-selectin CD62L is associated with the neutrophil maturation and activation status (Pillay et al., 2012a). Therefore, CD62L expression on blood and tumor neutrophils was assessed by flow cytometry to further characterize the neutrophilic phenotype (Fig.4.20.).

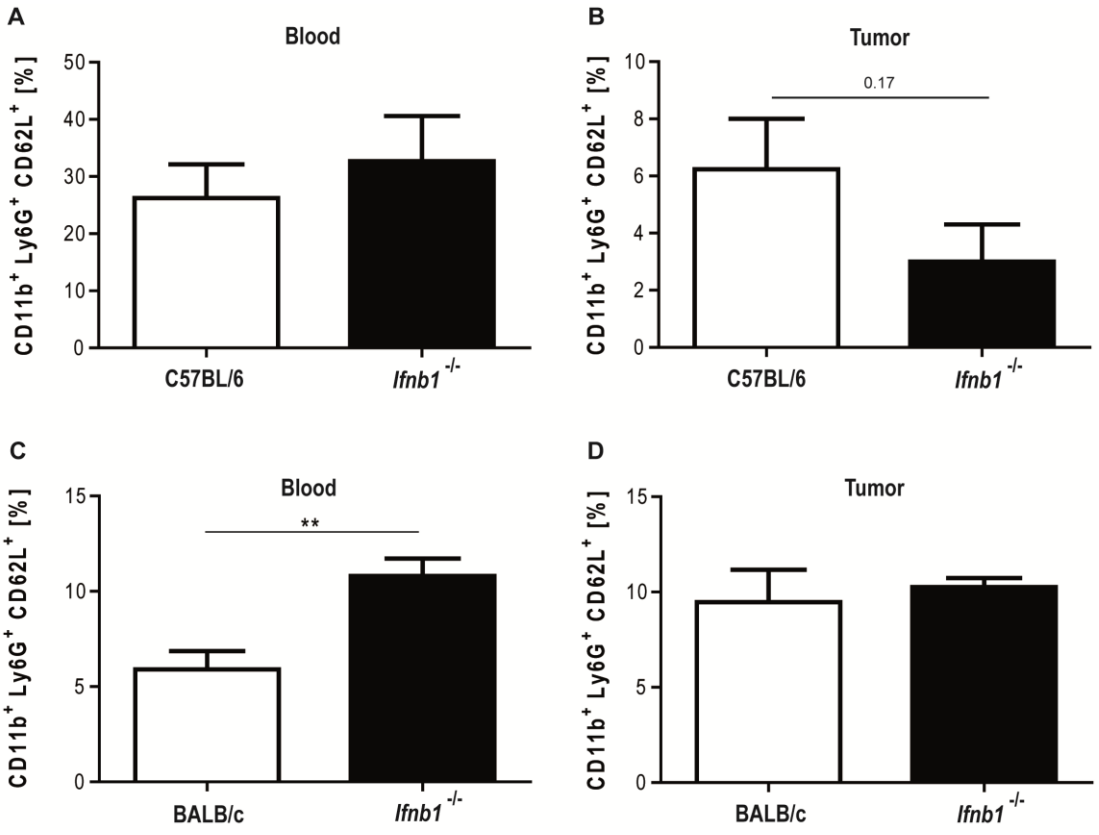

Figure 4.20. Accumulation of CD62L^{hi} neutrophils in the blood of *Ifnb1*$^{-/-}$ mice. To assess the expression of L-selectin on neutrophils, C57BL/6 and BALB/c mice, as well as syngeneic *Ifnb1*$^{-/-}$ animals, were challenged with B16F10 or 4T1 tumors, respectively. On day 14 after tumor inoculation single cell suspension of blood (A, B) and tumor (C, D) were prepared and analyzed for CD62L expression on CD11b^{+} GR1^{+} neutrophilic granulocytes via flow cytometry. The experiment was repeated twice with at least five mice per group and statistical significance was calculated using unpaired students t test (*p≤0,05).

Consistent with the observed accumulation of immature neutrophils in circulation of tumor bearing *Ifnb1*$^{-/-}$ animals (Fig. 4.8.), a significantly increased percentage of CD62L^{+} neutrophils was detected in the blood of tumor bearing BALB/c mice deficient for endogenous IFN-β (Fig.4.20 C). The same effect was observed for C57BL/6 mice, but the difference was statistically not significant (Fig.4.20. A). Interestingly, once extravasated into the tumor tissue, CD62L expression did apparently not depend on presence or absence of endogenous IFN-β any more (Fig.4.20. B and D).

4.3.3. TAN do not secret arginase 1 or suppress T cell proliferation

In contrast to the immunostimulatory functions of anti-tumor N1 neutrophils, pro-tumor N2 neutrophils were reported to exert immunosuppressive function towards T cells (Mayadas et al., 2014). Such TANs were shown to produce arginase 1 (Fridlender and Albelda, 2012) or inhibit T cell responses by the secretion of ROS in a cell contact dependent manner via Mac-1 (Pillay et al., 2012b). To test whether the ability of neutrophils to produce arginase 1 is influenced by endogenous IFN-β, C57BL/6 and syngeneic *Ifnb*1$^{-/-}$ mice were challenged with B16F10 melanoma. On day 14 after tumor inoculation arginase 1 content in blood (Fig.4.21. A) or tumor (Fig.4.21. B) derived neutrophils was assessed via flow cytometry.

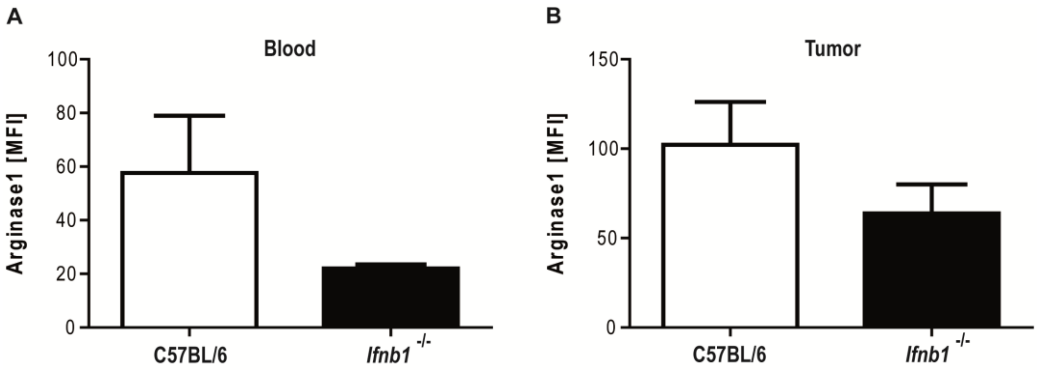

Figure 4.21. Arginase 1 production of neutrophils from blood and tumor in presence or absence of endogenous IFN-β. To assess the arginase 1 content of neutrophils from different sources, C57BL/6 and syngeneic *Ifnb1*$^{-/-}$ animals were challenged with B16F10 melanomas. On day 14 after tumor inoculation single cell suspension of blood (A,) and tumor (B) were prepared and analyzed for arginase 1 expression of CD11b^{+} GR1^{+} neutrophilic granulocytes via flow cytometry. The experiment was repeated twice with at least five mice per group.

Apparently, no significant differences in neutrophils from the different anatomical compartments were observed in presence and absence of endogenous IFN-β. The mean fluorescence intensity detected for neutrophilic arginase 1 was generally very low and close to the detection limit.

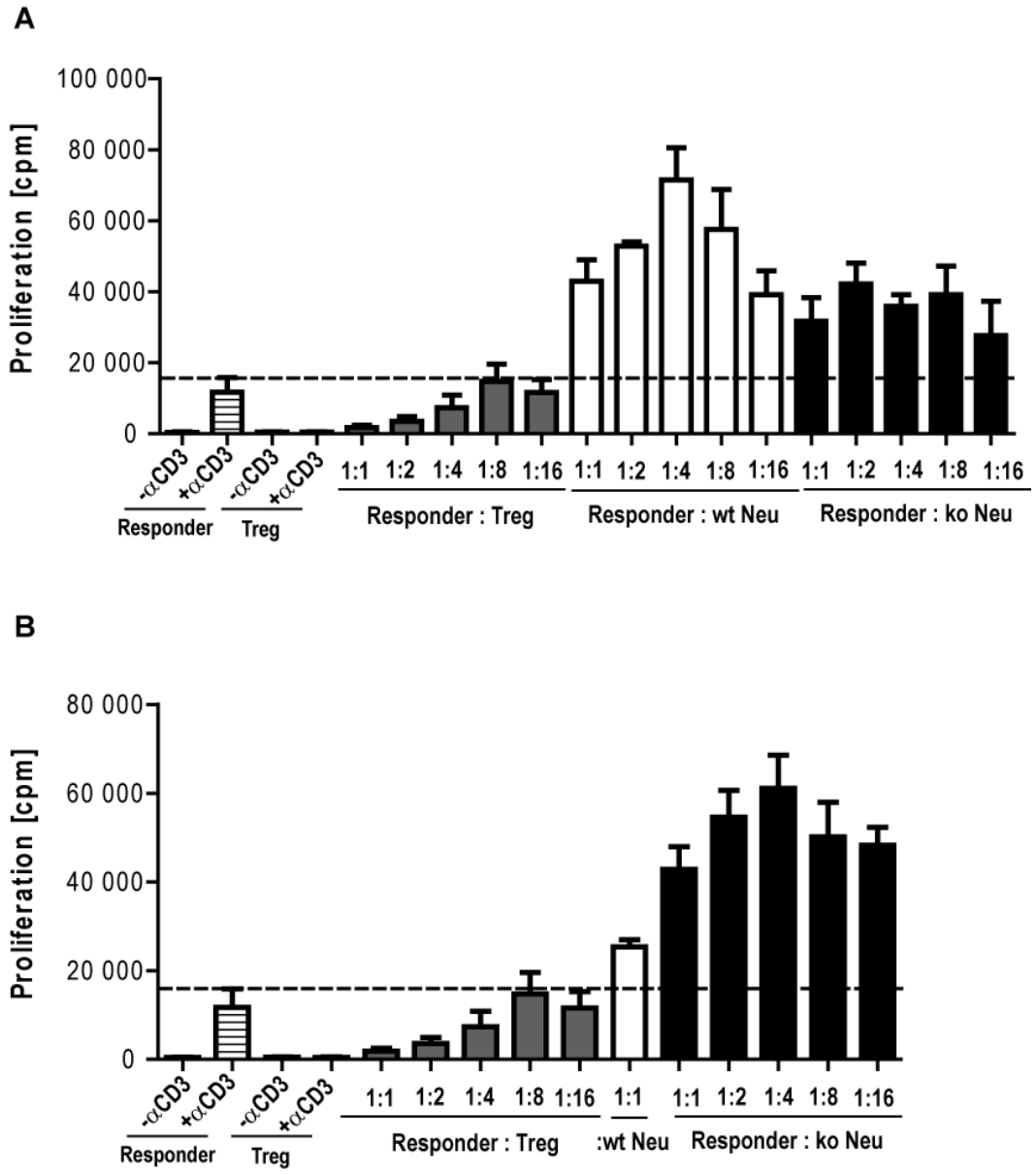

Figure 4.22. Neutrophils from tumor bearing mice show no T cell suppressive capacity. To assess suppressive activities of neutrophils $CD4^{+}CD25^{-}$ αCD3 stimulated responder T cells from BALB/c mice were co-cultivated in different ratios with $CD4^{+}CD25^{+}$ regulator T cells derived from BALB/c mice or sorted neutrophils from blood (A) or tumors (B) of wild type or *Ifnb1*$^{-/-}$ animals. T cell proliferation rate was assessed after 72h by ^{3}H-thymidine incorporation. The experiment was repeated 3 times.

In line with this, neutrophils sorted from blood (Fig.4.22. A) or tumor (Fig.4.22. B) of wild type and syngeneic *Ifnb1*$^{-/-}$ mice failed to suppress CD3 mediated T cell proliferation. Instead, neutrophil co-culture stimulated T cell proliferation and neither anatomical compartment nor genotype of the respective granulocytes abrogated this effect.

4.3.4. Reduced killing capacity of neutrophils in *Ifnb1*$^{-/-}$ mice

Neutrophils, besides their immunoregulatory functions, have the capacity to directly kill tumor cells (Hicks et al., 2006). This ability is remarkably reduced in N2 TANs (Fridlender and Albelda, 2012) and it is unclear which factors influence this process. Therefore, neutrophil cytotoxicity against tumor cells was tested in tumor bearing mice deficient or sufficient for IFN-β. To this end, neutrophils derived from blood and tumor of BALB/c (Fig.4.23. A), C57BL/6 (Fig.4.23. B) or syngeneic *Ifnb1*$^{-/-}$ animals were sorted and co-cultivated with luciferase expressing 4T1 tumor cells at an effector-target ratio of 2:1 for 18 hours with or without the addition of rmIFN-β. The luciferase activity of living tumor cells was subsequently measured using the IVIS 200 *in vivo* imaging system.

Indeed, cytotoxicity against tumor cell was significantly reduced in neutrophils sorted from blood (Fig.4.23. A) and tumor (Fig.4.23. A and B) of *Ifnb1*$^{-/-}$ animals. However, addition of low amounts of rmIFN-β to the culture medium completely restored the killing capacity of *Ifnb1*$^{-/-}$ neutrophils. Interestingly, a general reduction of neutrophilic killing ability was detected for TANs in comparison to blood derived neutrophils, indicating further influences of the tumor microenvironment on neutrophilic functions.

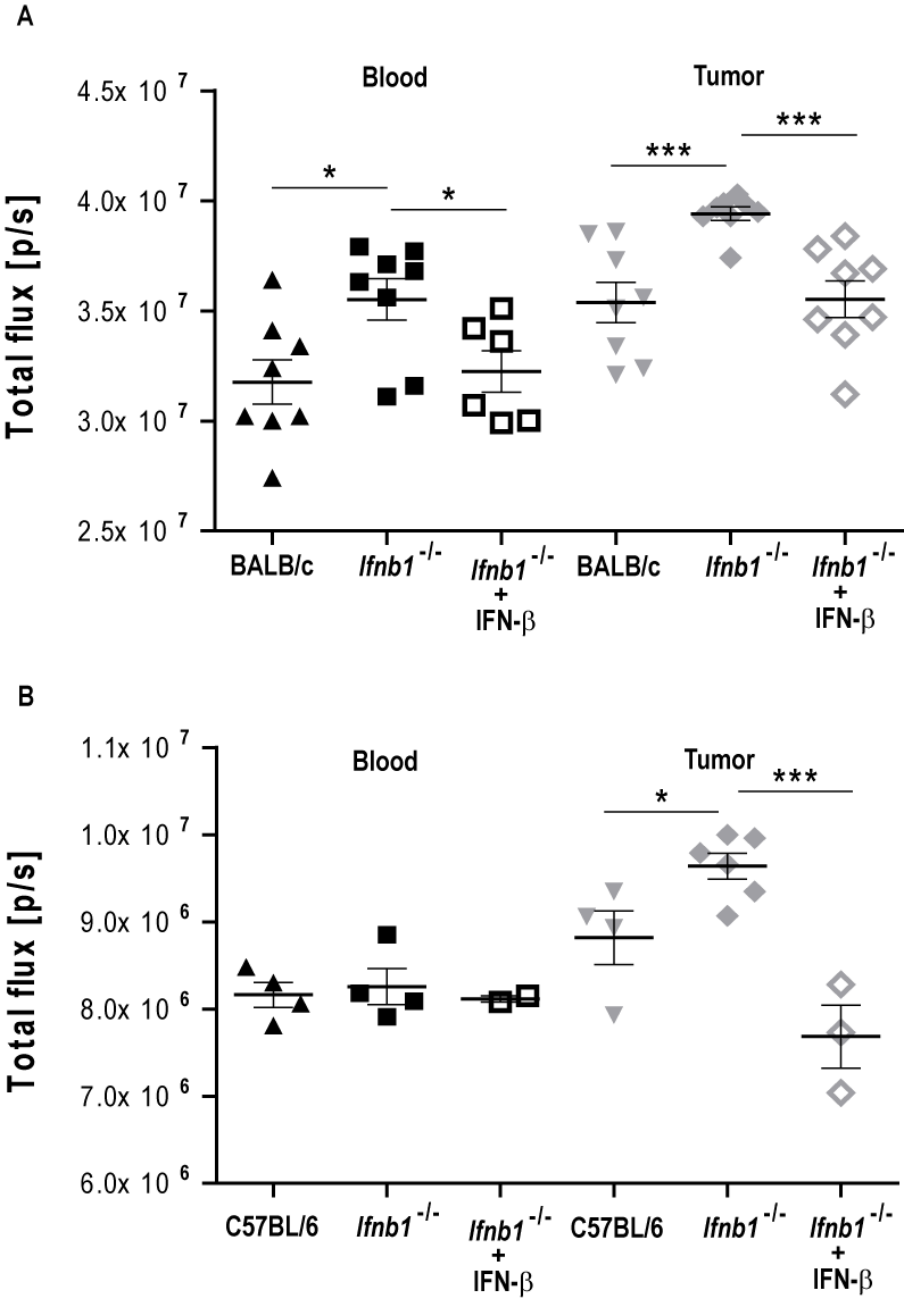

Figure 4.23. Reduced killing capacity of neutrophil granulocytes in the absence of endogenous IFN-β. To evaluate the ability of neutrophils from tumor bearing mice to kill tumor cells, BALB/c (A) and C57BL/6 (B) mice as well as syngeneic *Ifnb1*$^{-/-}$ animals were challenged with 4T1 or B16F10 tumors, respectively. On day 14 after tumor inoculation neutrophils from blood and tumors were sorted and co-incubated with luciferase expressing 4T1 tumor cells in an effector target ration of 2 to 1 for 18 h. Afterwards the luciferase activity of the residual living tumor cells was measured using an IVIS *in vivo* imaging system**.** The experiment was repeated twice, with at least five mice per group and statistical significance was calculated using unpaired students t test (*p≤0,05).

4.3.5. *Ifnb1*$^{-/-}$ tissue neutrophils produce decreased amounts of TNF-α

In 1975 Carswell, Old and colleagues identified an endo-toxin induced serum factor that induces tumor necrosis and named it TNF-α (Carswell et al., 1975). Nowadays, this cytokine is known as a major regulator of inflammation that can exert both, pro-tumor as well as anti-tumor functions, depending on its concentration (Balkwill, 2009). Importantly, the tumor toxic effect of TNF-α is not only due to direct cytotoxic or cytostatic effects on the tumor cells, but can also be attributed to its immunostimulatory and vessel disrupting functions (Daniel and Wilson, 2008). Of note, one important feature of N1 anti-tumor neutrophils is the local production of TNF-α at tumor site (Fridlender and Albelda, 2012).

Therefore, to further elucidate the impact of endogenous IFN-β on TAN polarization, neutrophilic TNF-α production was assessed in different anatomical compartments of tumor bearing C57BL/6 as well as BALB/c mice and syngeneic *Ifnb1*$^{-/-}$ animals (Fig.4.24).

In both tumor models applied, no differences regarding the percentage of TNF-α producing blood neutrophils were detected (Fig.4.24. A and B). However, neutrophilic TNF-α production was remarkably increased after transmigration into target tissue. Most probably due to the activation of such cells (Fig.4.24. C to E). This migration associated polarization appeared to be strongly influenced by endogenous IFN-β. Neutrophils from tumor (Fig.4.24. C) as well as lung (Fig.24.4. E and F) displayed a significantly reduced expression of TNF-α, in comparison to wild type controls. This was true for all mouse strains or tumor models tested.

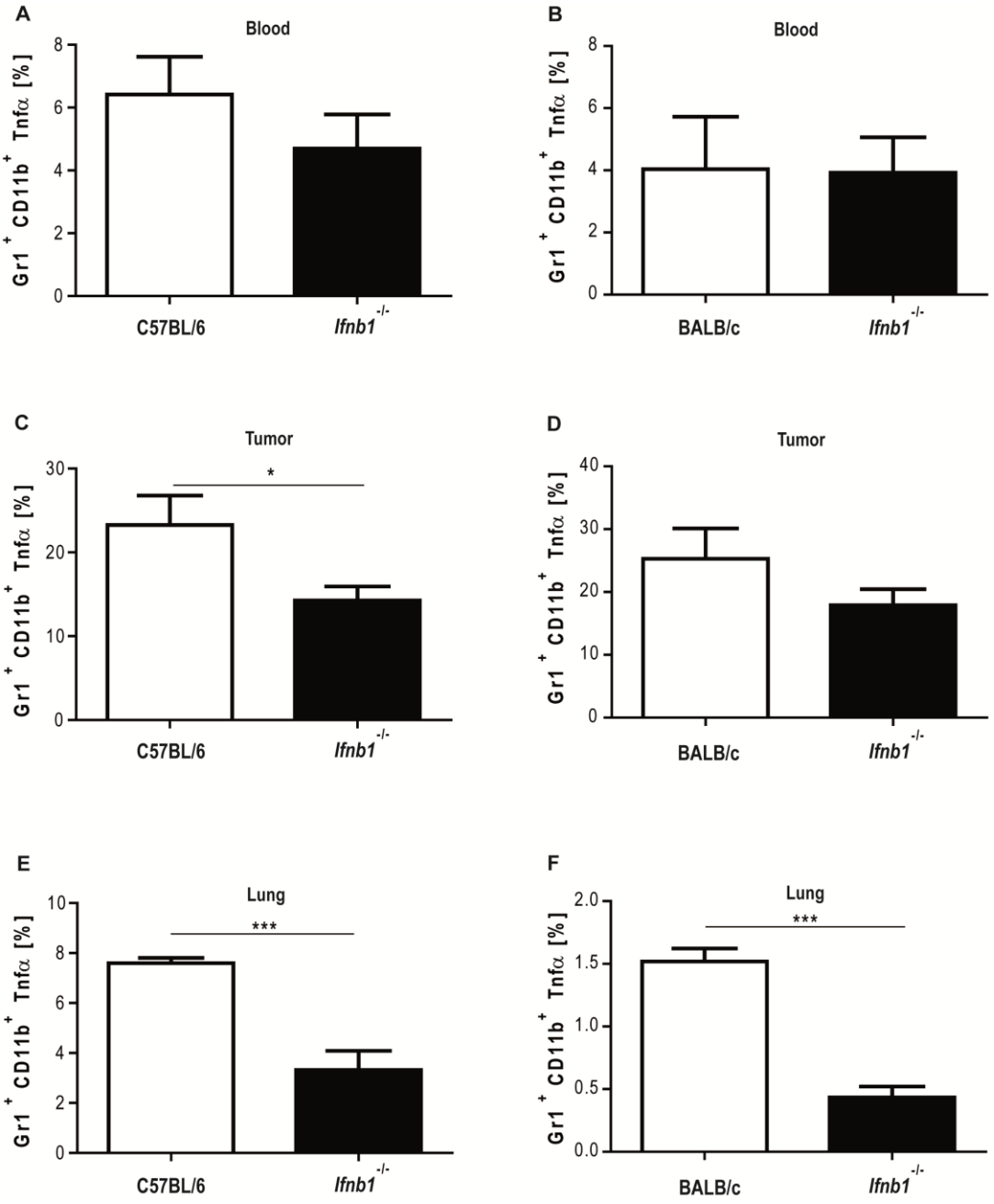

Figure 4.24 Decreased TNF-α production by neutrophils from tumor bearing *Ifnb1*$^{-/-}$ mice. To assess the production of TNF-α by neutrophils, C57BL/6 and BALB/c mice, as well as syngeneic *Ifnb1*$^{-/-}$ animals, were challenged with B16F10 or 4T1 tumors, respectively. On day 14 after tumor inoculation single cell suspension of blood (A, B), tumor (C, D) and lung (E, F) were prepared and analyzed for TNF-α expression in CD11b^{+} GR1^{+} neutrophilic granulocytes via flow cytometry. The experiment was repeated twice with at least five mice per group and statistical significance was calculated using unpaired students t test (*p≤0,05).

4.4. Influences of type I IFN tumor therapy on neutrophil polarization

Just shortly after the discovery of type I IFNs by Isaacs and Lindenmann (Isaacs and Lindenmann, 1987) the investigation of their therapeutic potential started. Nowadays, type I IFNs, i.e. IFN-α and IFN-β, are in clinical use for treatments of a broad variety of diseases, such as multiple sclerosis (Verweij and Vosslamber, 2013) or hepatitis C virus infections (Pawlotsky, 2013, 2014). In cancer therapy type I IFNs are also successfully applied for different tumor entities, including renal cell carcinoma or hairy cell leukemia (Wang et al., 2011) even though the exact mechanism of action is not clear to date. Besides direct cytostatic and cytotoxic effects of type I IFN on the tumor cells, several immunomodulatory effects are attributed to such treatments (Gajewski et al., 2012). Nevertheless, neutrophil polarization under type I IFN therapy has not been investigated thus far and therefore it became one of the aims of this thesis. Indeed, an effect of endogenous IFN-β on the polarization of tumor associated neutrophil granulocytes was elucidated. To additionally specify the effect of therapeutic IFN-β on neutrophil polarization, such cell isolated from tumor-bearing mice undergoing IFN-β treatment were phenotypically characterized.

4.4.1. Therapeutic intervention with type I IFNs induces an N1 anti-tumor neutrophil phenotype

High dose type I IFN therapy, as it is currently used in the clinics to treat different diseases, is often accompanied by severe side effects. This could negatively influence patient compliance and not seldom leads to the discontinuation of treatment (Kiladjian et al., 2011; Mocellin et al., 2013). Thus, it was of special interest to determine the effects of low dose type I IFN therapy on disease modifying aspects like immunosurveillance. Therefore, C57BL/6 as well as BALB/c mice and syngeneic *Ifnb1*$^{-/-}$ animals were challenged with B16F10 melanomas or 4T1 mammary carcinomas and treated i.v. with low doses of rmIFN-β. On day 14 after tumor inoculation neutrophilic phenotype was assessed by flow cytometry (Fig.4.25 and Fig.4.26.).

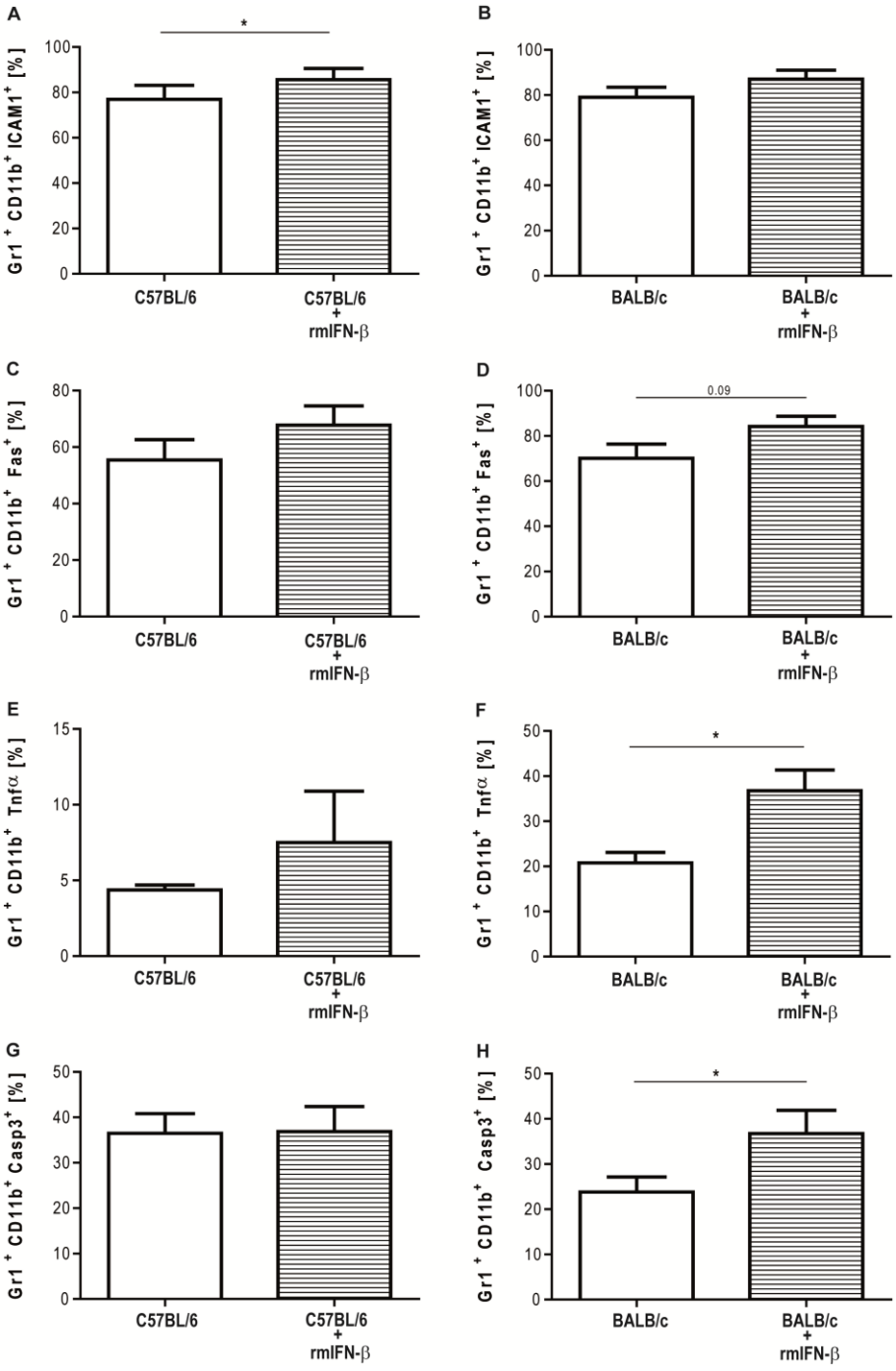

Figure 4.25. Phenotypic characterization of TAN upon low dose IFN-β therapy. C57BL/6 (A,C,E,G), BALB/c (B, D, F, H) wild type mice and syngeneic *Ifnb1*$^{-/-}$ animals were s.c. injected with B16F10 or 4T1 tumor cells, respectively. From day 3 after tumor inoculation onwards low dose IFN-β therapy was i.v. applied every other day. On day 14 after tumor inoculation single cell suspensions from tumor tissue were analyzed for the expression pattern of N1/N2 markers ICAM1, Fas, TNF-α and active caspase 3. The experiment was repeated at least once with five mice per group and statistical significance was calculated using unpaired students t test (*p≤0,05).

Type I IFN therapy induced neutrophil polarization towards an N1 like phenotype. On C57BL/6 background this was especially significant for the co-stimulatory cell-adhesion molecule ICAM1 (Fig.4.25. A). Also the other polarization marker tested showed a tendency of upregulation. This might indicate an N1 polarization as therapeutic effect of the IFN-β application (Fig.4.25. C, E and G). Especially the treatment of BALB/c mice resulted in the upregulation of the N1 marker TNF-α (Fig.4.25. F). Additionally, caspase 3 (Fig.4.25. H) was significantly upregulated upon type I IFN treatment. Moreover the extracellular N1 marker Fas (Fig.4.25. D) as well as ICAM1 (Fig.4.25. B) showed a slightly increased expression in response to the treatment.

4.4.2. Therapeutic polarization of neutrophils in the pre-metastatic lung

Metastasis is the spread of tumor cells from the primary lesion to another, non-adjacent organ. Importantly, multi organ failure due to metastasis causes 90% of the cancer associated deaths (Gupta and Massagué, 2006). Even though it would be highly desirable to directly target and control metastatic processes, the molecular mechanisms underlying this fatal devolution are largely elusive to date. Besides different tumor and target organ features that are known to determine the preferential metastatic sites, also the so called pre-metastatic niche is involved in the establishment of metastasis (Sceneay et al., 2013). Especially for lung metastasis, involvement of neutrophilic granulocytes in the formation of a permissive pre-metastatic niche has been demonstrated (Kowanetz et al., 2010). Type I IFNs seem to be involved in the regulation of this process (W & Andzinski et al. submitted). Therefore, the impact of a therapeutic intervention on lung neutrophil polarization using type I IFNs was characterized in the present work. C57BL/6 and BALB/c mice were challenged with B16F10 melanoma or 4T1 mammary carcinoma cells that are known to spontaneously metastasize to the lung (Parhar and Lala, 1987; Smith et al., 2004). Tumor bearing mice were treated with low amounts of rmIFN-β for a period of 14 days. Subsequently phenotypic polarization of lung neutrophils was analyzed using flow cytometry (Fig.4.26.).

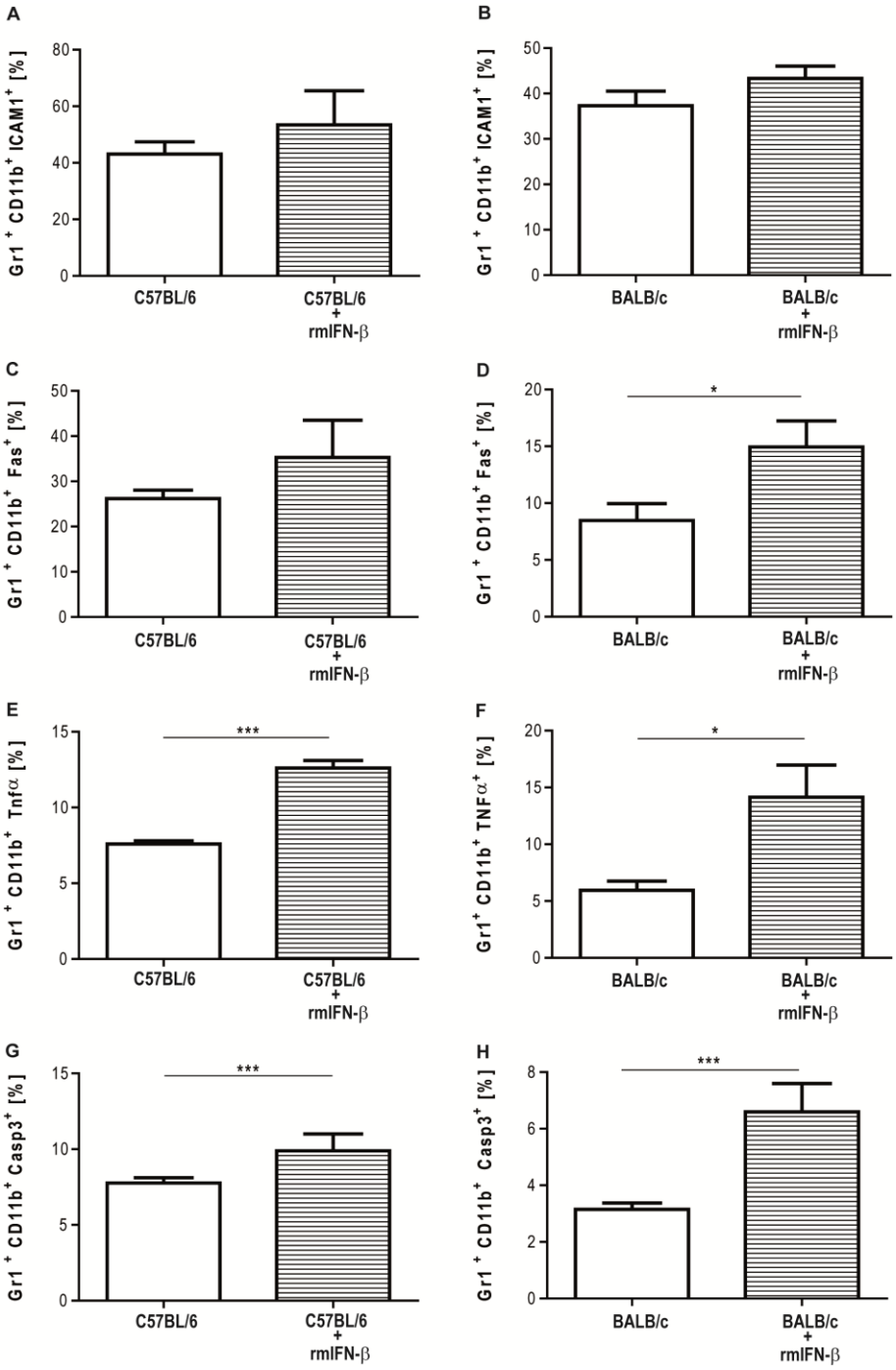

Figure 4.26. Phenotypic characterization of neutrophils in the pre-metastatic lung upon low dose IFN-β therapy. C57BL/6 (A,C,E,G) and BALB/c (B, D, F, H) wild type mice, as well as syngeneic *Ifnb1*$^{-/-}$ animals, were s.c. injected with B16F10 or 4T1 tumor cells, respectively. From day 3 after tumor inoculation low dose i.v. IFN-β therapy was applied every other day. On day 14 after tumor inoculation single cell suspensions from lung tissue were analyzed for the expression pattern of N1/N2 markers ICAM1, Fas, TNF-α and active caspase 3. The experiment was repeated at least once with five mice per group and statistical significance was calculated using unpaired students t test (*p≤0,05).

Regarding both tumor models, low dose IFN-β therapy clearly induced an N1 polarization of neutrophils in the pre-metastatic lung (Fig.4.26.). This was especially significant for the production of TNF-α (Fig.4.26. E and F) and the activity of effector caspase 3 (Fig.4.26. G and F) that were drastically upregulated upon treatment. Referring to the regulation of the N1 associated marker ICAM1 (Fig.4.26. A and B) and Fas (Fig.4.26. C and D) enhanced expression after therapeutic intervention was also detectable, indicating an influence of exogenous type I IFN therapy on neutrophil polarization in the pre-metastatic niche.

Taken together, these data demonstrate the high significance of therapeutic as well as endogenous IFN-β on the polarization of N1 anti-tumor TANs. In a long run this might influence the development of new therapeutic approaches targeting endogenous type I IFN induction to facilitate efficient immunosurveillance of cancer.

5. Discussion

The importance of neutrophils in the biology of cancer is well established now. Even though there are controversies regarding the differences between granulocytic myeloid-derived suppressor cells and TANs (Dumitru et al., 2012; Brandau et al., 2013). Especially their contribution to tumor angiogenesis, immunosurveillance and invasive growth became increasingly clear over the years (Jablonska et al., 2010; Fridlender et al., 2009; Gabrilovich et al., 2012b). Moreover, neutrophils represent an important prognostic marker in a broad variety of neoplasias. For instance, a high number of intra-tumoral neutrophils in localized, as well as metastatic renal cell carcinoma, correlates with a negative prognosis (Jensen et al., 2009). Similarly, infiltration by neutrophils has been correlated in gliomas with higher tumor stages (Fossati et al., 1999) and in pancreas carcinomas with more aggressive tumor phenotypes (Reid et al., 2011). In accordance, tumor infiltrating neutrophils represent a highly potent therapeutic target. Thus, understanding the physiology of tumor infiltrating neutrophils is of utmost importance.

The role of type I IFNs in this context was recently demonstrated. Higher numbers of neutrophils accumulate in tumors of mice that lack endogenous IFN-β. Such neutrophils not only produce higher amounts of pro-angiogenic factors, compared to their WT counterparts, but also secrete increased amounts of neutrophil attracting chemokines (Jablonska et al., 2010, 2013). The present work now adds further evidence for the major role of type I IFNs in the regulation of cancer immunosurveillance via the polarization of neutrophilic granulocytes and demonstrates for the first time a Sting dependent induction of IFN-β production by tumor infiltrating DCs.

5.1. IFN-β induction in solid tumors

The importance of endogenous type I IFNs in immunosurveillance of cancer is widely accepted and different approaches to abrogate type I IFN responses, like using neutralizing antibodies or knockout mice (*IFNAR*$^{-/-}$, *Ifnb1*$^{-/-}$), revealed significantly faster tumor growth and invasiveness of a broad variety of murine tumor models upon type I IFN deficiency (Gresser et al. 1990; Picaud et al. 2002; Gresser & Belardelli 2002; Jablonska et al. 2010).

This influence of type I IFN can be attributed to both, direct as well as indirect effects on the tumor cells. Hence, type I IFNs were demonstrated to increase the expression levels of MHC I (Maguire et al. 1990), enhance tumor antigen expression on the cell surface (Lindahl et al. 1973) or induce growth arrest and apoptosis in cancer cells (Bekisz et al. 2013; Takaoka et al. 2003).

In addition, endogenous type I IFNs exert diverse immunomodulatory functions. They are involved in cancer immunoediting (Dunn et al. 2005; Dunn et al. 2006), and activate innate immune cells e.g. NK cells (Gresser et al. 1994; Gill et al. 2011) or macrophages (Bekisz et al. 2013). Moreover, type I IFNs promote the adaptive anti-tumor response by suppressing regulatory T cells (Fradelizi & Gresser 1982), activating cytotoxic $CD8^+$ T cells (Harlin et al. 2009) as well as DCs (Bald et al. 2014) and enhancing humoral immunity (Le Bon et al. 2001). Despite these tremendous beneficial effects and the number of mechanisms described to date, some key aspects of type I IFN action during tumor progression are still unknown. Is constitutively produced type I IFN regulating all these phenomenons or is the IFN induced in growing tumors? If induced, which signaling pathways are involved and which cells contribute to the induced or constitutive IFN production?

The present work demonstrates type I IFN induction in different murine tumor models by using a very sensitive reporter mouse approach (Lienenklaus et al. 2009). However, other studies failed to detect induction of IFN-α/β in different spontaneous or transplantable tumor models (Gresser & Belardelli 2002). This controversy is likely due to the lower sensitivity of the methods used in the opposing papers. The luciferase reporter system in contrast can easily localize sites of IFN induction in a *Listeria monocytogenes* infection model where IFN-β is hardly detectable by ELISA (Solodova et al. 2011). Indeed, the IFN-β signal detected at tumor sites was relatively low, though clearly elevated above background IFN-β expression. It reached levels, typically detected in the thymus, the organ with the highest constitutive IFN-β expression (Lienenklaus et al. 2009).

Regarding the cellular source of type I IFNs at tumor site, the present results indicate that both, invading host cells as well as tumor cells, express IFN-β *in vivo*. Interestingly, no IFN-β was produced by tumor cells in cell culture. Thus, the tumor micromilieu *in vivo* apparently induces a type I IFN response in tumor cells. The signals however are still unclear and the importance of this tumor derived type I IFN in clinical settings remains to be determined. Of

note, in different human tumors such as glioblastoma multiforme (Sgorbissa et al. 2011) or malignant melanoma (Culig 2013; Lenci et al. 2012) mutations targeting the type I IFN system have been observed. Thus, it appears that during tumor development a counter selection against a functional type I IFN response takes place. To this end, the mechanisms and relevance of tumor cell derived type I IFN should be further investigated under physiological conditions in cancer patients.

Nevertheless, host cell derived type I IFN is of utmost importance in cancer surveillance (Gresser & Belardelli 2002). Here, by applying a tissue specific reporter mouse model (Solodova et al. 2011), the IFN-β producing cell population at tumor site could be revealed. It is characterized as $Tie2^{+}$ $LysM^{+}$ $CD11c^{+}$ $CD19^{-}$ and partly $CD4^{+}$. This indicates cells, that derive from hematopoietic precursors (Tie2) (Constien et al. 2001) and belong to the myeloid lineage (Hume 2011). Moreover, the cell population of interest expresses CD11c and a part expressed CD4 at least once in their life time. These observations suggest a population of infiltrating myeloid DCs (Shen et al. 2014; Haniffa et al. 2013) as cellular source of tumor-induced type I IFNs. Indeed, sorting of $CD11b^{+}$ $CD11c^{+}$ mDCs from tumor tissue in combination with subsequent qRT-PCR analysis revealed substantial IFN-β expression in such cells in comparison to splenic mDCs or B cells. This is in line with the observation, that IFN induction in the tumor is partially dependent on IRF3 and IRF5 as it has been previously described for IFN-β induction in DCs (Lazear et al. 2013).

In general, three major receptor systems can be involved in mounting a type I IFN response, namely TLRs, RLRs and CDRs (Gonzalez-Navajas et al. 2012). Crossing the IFN-β reporter mouse with knockout mutants for the respective adaptor proteins of these PRRs allowed to demonstrate that neither TLR nor RLR receptor signaling is responsible for tumor-induced type I IFN expression. The absence of the CDR signaling adaptor protein Sting (Keating et al. 2011; Paludan & Bowie 2013) however, substantially reduced IFN-β gene expression in tumor tissue. Therefore, it can be speculated that DNA of dying tumor cells might be the ligand inducing type I IFN expression in tumor tissue. Actually, it is known that cell death, for example induced by chemotherapeutic agents, can lead to the release of DAMPS that subsequently activate the immune system (Green et al. 2009). Furthermore, the anti-microbial peptide LL37, which is over expressed in psoriasis patients, very efficiently transports extracellular self-DNA into plasmacytoid DCs (Lande et al. 2007) or monocytes (Chamilos et

al. 2012) thus facilitating TLR or Sting mediated type I IFN induction, respectively. Moreover, TLR independent type I IFN induction has been reported upon phagocytosis of apoptotic cells by DNase II-deficient macrophages. Hence, the massive cell death in tumors accompanied by environmental conditions provoking defective DNA degradation in APCs is a potential mechanism mediating type I IFN induction in tumor infiltrating mDCs (Fuertes et al. 2013).

Sting was recently demonstrated to be involved in type I IFN induction upon DNA recognition. However, it is apparently not a direct DNA sensor (Keating et al. 2011). Further research is needed, to identify the receptor responsible for Sting mediated type I IFN expression. A possible candidate might be interferon inducible protein 16 (IFI16) which has been demonstrated to recruit Sting and initiate a TBK1-IRF3-dependant IFN-β induction in response to self and non-self DNA ligands (Unterholzner et al. 2010; Keating et al. 2011; Paludan & Bowie 2013). Interestingly, IFI16 is also known as tumor-suppressor exhibiting pro-apoptotic, anti-angiogenic as well as immunostimulatory effects (Mazibrada et al. 2010). Variation in constitutive IFI16 expression might be associated with human diseases (Choubey et al. 2008).

Recently, a loss-of-function variant of Sting was identified in humans (Jin et al. 2011). The resulting protein displays ≥90% decrease regarding its ability to stimulate IFN-β production in response to *Listeria monocytogenes* infection clearly emphasizing the relevance of Sting in humans. Importantly, the authors assume that approximately 3% of the American population is homozygous for this mutation. It would be very interesting to further investigate the impact of this genetic variant on tumor development especially in the light of the present study. Unfortunately no data on cancer associated deaths are available for this cohort so far.

Taken together the present findings regarding tumor-induced, Sting-IRF3/IRF5-mediated type I IFN expression in tumor infiltrating mDCs have important implications. They reveal potent targets for future cancer therapy and significantly improve our understanding of the molecular mechanisms underlying type I IFN modulated immunosurveillance of tumors.

5.2. IFN-β regulates the life span and polarization of neutrophils under tumor conditions

In WT tumor bearing mice, the live span of neutrophils is significantly shorter compared to neutrophils from IFN-β deficient animals. This is partially due to the fact that IFN-β is able to influence both the extrinsic as well as the intrinsic apoptosis pathways of neutrophilic granulocytes.

ROS production by neutrophils might play an important role in this context. For instance, a delayed rate of spontaneous neutrophil apoptosis was demonstrated in patients deficient for the enzyme NADPH oxidase (Kuijpers and Lutter, 2012; Kasahara et al., 1997). In line with this, it has been shown that hypoxia as well as pharmacological inhibition of NADPH oxidase and hydrogen peroxide scavengers are capable to decrease the rate of spontaneous neutrophil apoptosis (Lundqvist-Gustafsson and Bengtsson, 1999). The present data indicate that spontaneous production of ROS is diminished in the absence of endogenous IFN-β, thus potentially contributing to the delayed apoptosis of tumor infiltrating neutrophils in *Ifnb1*$^{-/-}$ mice and their accumulation in the neoplasia. The regulatory mechanism by which this cytokine influences the production of small molecules like ROS remains to be determined. Nevertheless, an influence of type I IFNs on ROS induced apoptosis has been demonstrated in hematopoietic cells deficient for the ISG UBP-43 (Yim et al., 2012). These cells are hypersensitive towards IFN-induced apoptosis and upregulate their ROS production after IFN treatment. Furthermore, the reduction of ROS by the treatment with ROS scavengers simultaneously reduced the type I IFN-mediated apoptosis in such cells (Yim et al., 2012). The present results confirm these findings. Lower numbers of neutrophils from blood and tumor became apoptotic when ROS were diminished.

Moreover, the death receptor Fas has been shown to be involved in spontaneous extrinsic cell death signaling in neutrophils (Liles et al., 1996). Even though Fas-ligand induced apoptosis is believed to be of no importance in steady state (Fecho et al., 1998), it has been demonstrated to be important under inflammatory conditions (Jonsson et al., 2005). Indeed, Fas has been shown to play a role in type I IFN-induced apoptosis in several types of neoplasias such as melanoma, multiple myeloma and chronic myeloid leukemia cells (Chawla-Sarkar et al., 2001; Selleri et al., 1997). Interestingly, the present work shows a significant reduction of Fas expressing neutrophils in tumors and lungs of *Ifnb1*$^{-/-}$ mice. This

is in line with the decreased apoptosis rate of neutrophils derived from tumor-bearing *Ifnb1*$^{-/-}$ mice. Thus, IFN-β dependent up-regulation of Fas in neutrophils might serve as a potential additional factor responsible for shortening the life span of pro-angiogenic neutrophils in tumors of normal animals.

Apoptosis is tightly regulated. In accordance, neutrophilic granulocytes constitutively express the pro-apoptotic Bcl-2 proteins Bax, Bak, Bid and Bik (Gabelloni et al., 2013). Especially Bax appears to play a major role in the regulation of neutrophilic life span, as *Bax*$^{-/-}$ mice show a reduced frequency of spontaneous apoptosis of such cells, compared to littermate controls (Gardai et al., 2004). In line with this, extended life span of neutrophils under inflammatory conditions is associated with reduced Bax expression (Dibbert et al., 1999). Under steady state conditions however, the activity of Bax is antagonized by anti-apoptotic Bcl-2 proteins such as BCL-xL (García-Sáez, 2012). The present study clearly demonstrates that the expression of anti-apoptotic BCL-xL was low under IFN sufficient conditions, while the pro-apoptotic factor Bax was up-regulated. In contrast, in the absence of endogenous IFN-β BCL-xL expression dominated over Bax, which is consistent with an extended life span of tumor infiltrating neutrophils in *Ifnb1*$^{-/-}$ mice. Thus, endogenous IFN-β drives the expression of Bax and BCL-xL in tumor infiltrating neutrophils towards a pro-apoptotic ratio. Consequently, a shorter life span of such cells is the result. Of note was the increased expression of both types of genes in tumor tissue, compared to blood. Apparently, after transmigration such genes are up-regulated and IFN-β is taking part in the fine tuning of this process.

As a direct consequence of mitochondrial membrane permeabilization and cytochrome c release into the cytoplasm, formation of the apotosome, including Apaf-1 oligomerization, is induced and passes in the activation of initiator caspase 9 (Li et al., 1997). Regarding neutrophil apoptosis, the intrinsic apoptosis pathway exhibits a low threshold requirement for cytochrome c, which is compensated by a high cytosolic expression of Apaf-1 (Murphy et al., 2003). In line with this, no differences in cytochrome c release comparing neutrophils of tumor bearing WT or *Ifnb1*$^{-/-}$ mice were detected in the present study. However, in the presence of IFN-β Apaf-1 gene expression was significantly upregulated. As consequence, significantly lower caspase 9 activity was observed in TANs in the absence of this cytokine.

Not only the expression of genes of the Bcl-2 family or Apaf-1 is regulated by IFN-β, but also expression of the effector caspase 3. Again a dramatic increase in expression level was observed in tumors compared to blood and the process was again strongly influenced by IFN-β. Extrapolating these data, one could speculate that the expression of a majority of members of the intracellular pathway leading to apoptosis are influenced by IFN-β.

The final executors of apoptosis, like caspase 3 (Chang and Yang, 2000), are expressed as pro-enzymes that need proteolytic cleavage to be activated. Besides the remarkable down-regulation of caspase 3 expression at the transcriptional level, the level of active caspase 3 was reduced in neutrophils derived from tumors of *Ifnb1*$^{-/-}$ mice. It is not clear whether IFN-β directly influences the proteolytic activation of this enzyme. More likely, the higher expression of the pro-enzyme at the transcriptional level in WT animals, results in generation of increased amounts of active caspase 3.

G-CSF has been identified as one of the major survival factors of neutrophilic granulocytes and is also involved in the regulation of neo-angiogenesis (Im et al., 2012). Moreover, G-CSF has been reported to reduce Bax expression (Dibbert et al., 1999), block its redistribution and restore its phosphorylation status thus leading to its inactivation. Via this mechanisms, G-CSF is capable to repress caspase activation (Maianski et al., 2004). In line with the inhibition of apoptosis and the prolonged neutrophil survival in the absence of endogenous IFN-β, a strong increase of G-CSF expression was observed in neutrophils from different anatomical compartments. Consistent with this, significantly elevated serum levels of G-CSF were detected in tumor bearing *Ifnb1*$^{-/-}$ mice. This might additionally explain the increased longevity of tumor infiltrating neutrophils. Moreover, an enhanced mobilization of immature neutrophils into circulation accompanied by a significantly faster turn over of these cells was detected in tumor bearing *Ifnb1*$^{-/-}$ mice in comparison to wild type controls. This might also be due to the increased G-CSF level in these animals. As such, the regulation of G-CSF expression by different molecular mechanisms is responsible for the control of neutrophil homeostasis (Mayadas et al., 2014). One possible way how endogenous IFN-β could be involved in this process would be via the regulation of IL-23 or IL-17 production. Especially the latter one is known to induce G-CSF and GM-CSF production by stromal cells and its expression can be induced by IL-23 (Croxford et al., 2012; Stark et al., 2005). Interestingly, IFN-β has been demonstrated to inhibit the production of IL-17 by CD4^{+} T cells (Chen et al.,

2009). In addition, the prolonged life span of neutrophils in the absence of endogenous IFN-β could be responsible for an increased production of IL-23 by tissue macrophages due to a reduced frequency of neutrophil phagocytosis that normally curbs the production of this cytokine and therefore regulates G-CSF secretion (Smith et al., 2007; Stark et al., 2005).

In conclusion, IFN-β is involved in the regulation of the life span, mobilization and turn over of pro-angiogenic neutrophils via a plethora of different pathways and mechanisms that involve synergistic regulation of genes directly participating in apoptosis, but also indirect pathways like cytokine production.

Importantly, most of the pro-apoptotic neutrophil features influenced by type I IFNs are also associated with N1 polarization of TAN. As such, a pro-apoptotic gene expression pattern including upregulation of the death receptor Fas (Fridlender et al., 2009) as well as an enhanced production of cytotoxic ROS (Zivkovic et al., 2007) are important characteristics of anti-tumor neutrophils. Further evidence for N1 neutrophil polarization by type I IFN is given by its capacity to facilitate the expression of co-stimulatory molecules like ICAM1, that was shown to be upregulated in N1 anti-tumor neutrophils upon tissue migration and activation (Piccard et al., 2012). ICAM1 is especially important for T cell activation under conditions that provide low co-stimulation via CD80 and CD86 (Lebedeva et al., 2005). It can induce the activation of cytotoxic $CD8^+$ T cells (Chen et al., 1999) as well as repress the secretion of immunosuppressive IL-10 by activated $CD4^+$ T cells (Labuda et al., 1998). Thus, regulation of ICAM1 expression by endogenous IFN-β could be involved in tumor immunosurveillance.

Another characteristic of N1 neutrophils is the secretion of TNF-α (Fridlender and Albelda, 2012) that was remarkably reduced in the absence of endogenous IFN-β, suggesting N2 polarization of neutrophils. Indeed, anti-tumor neutrophils have been demonstrated to activate DCs via the secretion of TNF-α (van Gisbergen et al., 2005a). Importantly, TNF-α has additionally been demonstrated to induce neutrophil apoptosis (Ferrante, 1992). Therefore elevated production of TNF-α by N1 neutrophils is in line with the observed shorter life span of such cells and IFN-β can apparently influence this process.

Similarly, the most prominent anti-tumor neutrophil feature, i.e. their ability to directly kill tumor cells (Gerrard et al., 1981; Zivkovic et al., 2007; Fridlender et al., 2009), appears to be controlled by IFN-β. Neutrophils isolated from blood or tumors of *Ifnb1*$^{-/-}$ mice displayed a significantly reduced tumor killing capacity in comparison to wild type controls, independent

of mouse strain and tumor model. This is even more eminent, as the addition of rmIFN-β into the culture completely rescued the function, indicating the possibility to re-polarize neutrophils by therapeutic intervention. The molecular mechanism potentially involved at this point could be the decreased neutrophilic ROS production due to IFN-β deficiency. As such, oxidative damage caused by singlet oxygen has been demonstrated to mediate tumor cell lysis (Lichtenstein et al., 1989; Zivkovic et al., 2005). In line with this, N1 neutrophil polarization in response to TGF-β antagonists induced tumor toxicity via enhanced ROS production (Fridlender et al., 2009).

Although, some studies attribute T cell suppressive properties to N2 TAN (Fridlender et al., 2009; Kousis et al., 2007; Di Carlo et al., 2001), other authors use this capacity to define granulocytic MDSCs (Gabrilovich and Nagaraj, 2009; Gabrilovich et al., 2012a). However, the neutrophil population characterized during the present work, did neither display the ability to repress T cell proliferation nor did it produce arginase 1, notwithstanding the pro-tumorigenic function of such cells (Jablonska et al., 2010, 2013).

Besides the IFN-β dependency of N1 neutrophil characteristics demonstrated in the present work, the pro-angiogenic expression of c-myc, MMP-9 and VEGF (Jablonska et al., 2010) as well as the secretion of auto-attracting CXC chemokines (Jablonska et al., 2013) seem to be regulated by endogenous type I IFNs. Importantly, also in these experiments additional rmIFN-β was able to reconstitute wild type gene expression. To this end, therapeutic usage of IFN-β was tested for its effect on neutrophil polarization in the present study. Indeed, type I IFN therapy augmented several N1 characteristics of neutrophils associated to the primary tumor and the pre-metastatic lung. Of note, this was independent of mouse strain or tumor model. It apparently influenced neutrophils accumulating in the pre-metastatic niche to a higher extend than those associated with the primary lesion. This is most probably due the comparably low amounts of IFN-β and the intravenous route of application used during the present work that preferentially supply strongly vascularized organs like the lung with therapeutic relevant concentrations of the cytokine. Anyway, agents abrogating the formation of a permissive pre-metastatic niche could be of substantial benefit for the clinical management of high risk patients. Nevertheless, future research on the therapeutic use of type I IFNs should carefully consider its effect on neutrophils.

5.3. Clinical relevance and concluding remarks

Even though, type I IFNs such as IFN-α and IFN-β have been successfully used to treat different subtypes of solid tumors and hematologic malignancies, the exact molecular mechanisms of their action are still not clear (Wang et al., 2011). Besides direct cytostatic and cytotoxic effects (Murata et al., 2006) several beneficial immunomodulatory effects on NK cells, monocytes, macrophages, DCs, T cells and B cells have been attributed to type I IFNs (Bekisz et al., 2013). However, data linking type I IFN therapy and modulation of TAN phenotype are not available so far. The experiments performed during the present study clearly demonstrated a therapeutic effect of rmIFN-β on neutrophil polarization under tumor conditions. More precisely, a clear shift towards an N1 like TAN phenotype was detected under low dose IFN-β therapy in all mouse models applied, identifying neutrophils as important target cells of this treatment.

Neutrophils themselves were recently identified as effector cells during antibody-mediated tumor therapy (van Egmond, 2008), gene-therapy (Souto et al., 2011) and therapeutic application of pathogen derived biologics for cancer treatment (Galluzzi et al., 2012a; Kandasamy et al., 2011). Especially the latter ones, such as Coley toxin or bacillus Clamet-Guérin (BCG), are known to potently activate TLR signaling and induce a type I IFN mediated immune response (Kuchtey et al., 2006). Noteworthy, the connection of neutrophil and type I IFN effects regarding these therapies has not been investigated up to now. Obviously, further research on type I IFN inducing compounds could possibly help to identify potent anti-tumor agents acting on this basis.

In conclusion the present work elucidates additional mechanisms controlling neutrophil polarization and highlights the tremendous therapeutic potential of this process. Moreover, it generates further insight to the underlying pathways responsible for tumor-induced type I IFN expression. In the long run this knowledge might be used to directly target and activate endogenous type I IFN production during cancer immunotherapy, for example to prevent tumor metastasis in an adjuvant situation or boost the therapeutic effects of anti-tumor therapies such as irradiation (Burnette et al., 2011). Additionally, determination of genetic variants regarding type I IFN induction and response in tumor patients could be used in terms of personalized medicine, for example to predict the outcome of certain therapies.

All in all, further research efforts regarding this topic will definitely lead to applicable new treatment strategies of diverse malignancies.

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7. Appendices

7.1. Abbreviations

APCs – antigen presenting cells

APC- allophycocyanin

BM – bone marrow

CDA – cytosolic DNA receptor

cDCs – conventionsal dendritic cells

DCFDA - 2´, 7´-dichlorfluorescein diacetate

DCs – dendritic cells

EDTA – ethylendiaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

ER – endoplasmatic reticulum

FACS – fluorescence activated cell sorting

FCS – fetal calf serum

Fig. – figure

FITC – fluorescein isothiocyanate

FSC – forward scatter characteristics

i.e. – this is

IFN – interferon

IL – interleukin

IMDM – Iscove´s modified Dulbecco´s medium

i.v. – intra venous

LPS – lipopolysaccharide

MFI – mean fluorescence intensity

MHC – major histocompatibility cmplex

PAMPs – pathogen associated molecular patterns

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PE – phycoerythrin

PI – propidium iodide

PRR – pathogene recognition receptors

RIG - retinoic acid inducible gene

SSC – side scatter characteristics

TLR – toll like receptor

WT – wild type

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